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(21) International Application Number: PCT/US98/17991 (22) International Filing Date: 18 September 1998 (18.09.98) (30) Priority Data: 08/933,149 18 September 1997 (18.09.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/933,149 (CIP) Filed on 18 September 1997 (18.09.97) (71) Applicant (for all designated States except US): WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WATSON, Mark, A. [US/US]; 1461 Meadowside Drive, St. Louis, MO 63146 (US). FLEMING, Timothy, P. [US/US]; 743 Eaglebrooke Drive, St. Louis, MO 63021 (US). (74) Agents: HENDERSON, Melodie, W. et al.; Howell & Haferkamp, L.C., Suite 1400, 7733 Forsyth Boulevard, St. Louis, MO 63105-1817 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims.</i>
(54) Title: MAMMAGLOBIN, A SECRETED MAMMARY-SPECIFIC BREAST CANCER PROTEIN (57) Abstract <p>A purified and isolated DNA sequence and the encoded mammary-specific secreted protein, mammaglobin, are disclosed. Also disclosed are methods for detecting breast cancer based upon the overexpression and secretion of mammaglobin by breast cancer cells. The methods detect and/or quantitate the presence of mammaglobin or the mRNA encoding mammaglobin. Immunotherapy-based methods for treating a breast cancer patient with a mammaglobin-expressing tumor are also disclosed. The methods involve using mammaglobin antigens to induce a humoral and/or cell-mediated immune response against the tumor.</p>		

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MAMMAGLOBIN, A SECRETED
MAMMARY-SPECIFIC BREAST CANCER PROTEIN

Related Applications

This application is a continuation-in-part of PCT/US96/08235, filed May 31, 1996 which is a continuation-in-part of U.S. Application No. 08/455,896, filed
5 May 31, 1995.

Background of the Invention

(1) Field of the Invention

This invention relates generally to the field of breast cancer pathogenesis and, more particularly, to a cDNA sequence and encoded mammary-specific protein
10 for use in detecting and treating breast cancer.

(2) Description of the Related Art

Breast cancer is one of the most common and potentially lethal of cancers.

Although early diagnosis and treatment can reduce morbidity and mortality related to the disease, the positive predictive value of mammography has
15 been estimated to be only about 25% (Hall et al., *N Engl J Med* 327:319-328, 1992 which is incorporated by reference). It would, therefore, be desirable to have a means for detecting the cancer earlier than the cancer can be detected using mammography and a genetic or biochemical marker might be able to provide such means to complement and increase the predictive value of mammography. (Hayes, *Hematol*
20 *Oncol Clin N Am* 8:485, 1994 which is incorporated by reference).

The development of breast cancer is accompanied by a number of genetic changes (For review see Porter-Jordan, *Hematol Oncol Clin N Am* 8:73, 1994 which is incorporated by reference). Such changes include gross chromosomal alterations and loss of genetic markers (Devilee et al, *Biochim Biophys Acta* 1198:113, 1994;
25 Callahan et al, *J Cell Biochem Suppl* 17:167, 1993 which are incorporated by reference). The progression of breast neoplasia has also been shown to result in qualitative and quantitative changes in expression of previously identified genes that encode growth factors and their receptors (Zajchowski et al., *Cancer Res* 48:7041, 1988 which is incorporated by reference), structural proteins (Trask et al., *Proc Natl*

Acad Sci 87:2319, 1990 which is incorporated by reference), second messenger proteins (Ohuchi et al., *Cancer Res* 26:2511, 1986 which is incorporated by reference), and transcription factors (Harris, *Adv Cancer Res* 59:69:1992 which is incorporated by reference). These changes in gene expression could potentially form the basis for developing a breast cancer marker, although the precise role of these gene changes in the pathogenesis of breast carcinoma in patient biopsy samples is not well understood.

In addition to providing a genetic or biochemical marker for breast cancer for early detection of the disease, it would also be desirable to have a tumor marker that might provide an estimation of prognosis, a means for selection and evaluation of therapy and a means for the targeting of therapy. Although a number of tissue markers have been identified, none are sufficiently sensitive or tumor specific to be ideally suited for diagnosis or for screening the general population. (*Id.*). Thus, there remains a continuing need for a breast cancer marker such as a gene along with its expressed protein that can be used to specifically and selectively identify the appearance and pathogenic development of breast cancer in a patient, and that can be used in tumor-specific immunotherapy.

Using a modified differential display polymerase chain reaction technique to isolate differentially expressed sequence tags from mammary carcinoma, several sequence fragments were isolated that were uniquely expressed in neoplastic mammary epithelial tissue as compared to normal tissue controls (Watson and Fleming, *Cancer Res* 54:4598-4602, 1994 which is incorporated by reference). The discovery of one of these sequence tags identified as DEST002 has led to the discovery and isolation of the novel full length cDNA and encoded protein now referenced as mammaglobin. The cDNA and protein are both new.

Summary of the Invention

Briefly, therefore, the present invention is directed to the identification of novel genes whose expression is increased in breast cancer and to the isolating of cDNA's from the mRNA's of these genes. Accordingly, applicants have succeeded in discovering a novel cDNA and the encoded mammary-specific secretory protein, mammaglobin. The cDNA is in purified and isolated form and has a nucleotide sequence identified as SEQ ID NO:1 and the encoded protein, mammaglobin, is in

purified and isolated form and has an amino acid sequence identified as SEQ ID NO:2.

Mammaglobin is overexpressed in 27% of stage I primary breast cancer tumors. This suggests that dysregulation of the mammaglobin gene occurs early and frequently in breast cancer. The discovery of mammaglobin and its cDNA, therefore, provide the basis for the development of novel methods and compositions for the detection and treatment in humans and other mammals.

Thus, the present invention is directed to novel methods for detecting the presence of breast neoplasia cells in a sample. In one embodiment, cDNA encoding mammaglobin or a derivative of said cDNA is used to detect the presence of mammaglobin mRNA in a sample. The method comprises the steps of: (a) providing a polynucleotide containing a nucleotide sequence having the sequence of SEQ ID NO:1 or a derivative thereof, (b) incubating the nucleotide sequence with the sample under conditions in which the sequence can hybridize with mRNA from breast neoplasia cells, and (c) detecting the existence of a DNA-RNA hybridization complex.

Another aspect of the present invention provides a kit for detecting the presence of breast neoplasia cells in a sample by hybridization. The kit comprises a polynucleotide containing a nucleotide sequence having the sequence of SEQ ID NO:1 or a derivative thereof packaged in a container.

In another embodiment of the present invention, mammaglobin expression in a sample is determined by detecting the presence of cDNA that is reverse transcribed from mammaglobin mRNA in the sample. The method comprises the steps of: (a) producing a cDNA encoding mammaglobin from mRNA using the reverse transcription method in a sample obtained from a patient, (b) providing two primers for the polymerase chain reaction method which comprise oligomers that flank or lie within the cDNA encoding mammaglobin, and (c) amplifying the cDNA encoding mammaglobin by the polymerase chain reaction method. The two primers have nucleotide sequences comprising SEQ ID NO:3 and SEQ ID NO:4.

Another embodiment to the present invention provides a kit for detecting the presence of breast neoplasia cells in a sample by the polymerase chain reaction. The kit comprises two primers for the polymerase chain reaction method which

comprise oligomers that flank or lie within a cDNA encoding mammaglobin packaged in a container. The two primers have nucleotide sequences comprising SEQ ID NO:3 and SEQ ID NO:4.

In another embodiment of the present invention, the presence of
5 mammaglobin protein expressed by a tumor cell is detected in a sample using specific antibodies to the mammaglobin protein. The specific antibodies can be polyclonal or monoclonal antibodies.

The invention is also directed to novel compositions and methods for treating breast neoplastic disease using mammaglobin antigens capable of inducing an
10 antibody-mediated and/or a cell-mediated, i.e., through activated T cells, immune response against a mammaglobin-expressing tumor.

One embodiment of a composition according to the invention comprises a mammaglobin B cell antigen capable of activating mammaglobin-specific B cells. The B cell antigen comprises a mammaglobin-specific B cell epitope and a T_H
15 epitope, or determinant, recognized by T helper cells.

In another embodiment, the mammaglobin antigen is a mammaglobin T_C cell antigen recognized by mammaglobin-specific cytotoxic T lymphocytes which comprises a T_C cell epitope and a binding site, or agretope, for a MHC class I molecule.

20 Yet another embodiment of a composition according to the invention comprises B cell and T_C cell antigens.

Methods for treating a patient with a mammaglobin-expressing tumor include adoptive immunotherapy, which comprises *ex vivo* stimulation with a mammaglobin antigen of mammaglobin-specific lymphocytes isolated from the
25 patient and subsequent administration of the activated lymphocytes to the patient, and *in vivo* stimulation of an anti-mammaglobin immune response, which comprises administering to the patient a vaccine comprising a mammaglobin antigen.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of a nucleotide sequence and
30 encoded amino acid sequence that can serve as markers for breast cancer cells; the provision of methods for early detection of the presence of breast neoplasia cells; the provision of means for detecting breast cancer that can complement mammography

and increase the predictive value; the provision of methods that can provide an estimation of prognosis; the provision of markers that will allow the targeting of therapy; and the provision of compositions for stimulating a cellular and humoral immune response against the tumor.

5 Brief Description of the Drawings

Figure 1 illustrates the strategy used to isolate the full length mammaglobin cDNA including the Rapid amplification of cDNA Ends (RACE) Polymerase Chain Reaction (PCR) technique and subsequent subcloning into vectors pGEM7Z and pCEV27.

10 Figure 2 illustrates the human cDNA sequence of SEQ ID NO:1 (nucleotides numbered above) and the amino acid sequence of the encoded mammary-specific protein, mammaglobin (SEQ ID NO:2)(amino acids numbered below), the solid bar indicating the 403 bp fragment (SEQ ID NO:5) isolated by the RACE PCR method and the open bar indicating the 206 bp DEST002 sequence (SEQ
15 ID NO:6);

Figure 3 illustrates the amino acid sequence of the mammary-specific protein, mammaglobin (hMAM), (SEQ ID NO:2) compared to rat prostatic steroid binding protein subunit C3 (rPSC3)(SEQ ID NO:7) and human clara cell 10 kD protein (hCC10)(SEQ ID NO:8) with identities marked by bold letters and double
20 lines and structurally similar amino acids marked by single lines;

Figure 4 illustrates (A) the Northern blot analysis of hybridization of the human cDNA sequence encoding the mammary-specific protein, mammaglobin (hMAM), to mRNA expressed by tissues from breast neoplasia, normal breast and other adult tissues and (B) the analysis of RT/PCR amplified samples of tissues from
25 breast neoplasia, normal breast and other adult tissues;

Figure 5 illustrates the translation of the mammary-specific cDNA sequence in an *in vitro* rabbit reticulocyte lysate assay system;

Figure 6 illustrates Northern blot hybridization with the cDNA encoding mammaglobin showing detection of mRNA in tumor 2410, in tumors from three of
30 eight other patients (shown in bold), and to a lesser extent, in normal breast tissue (shown in italics), and comparing in two cases (the four lanes on the right) mammaglobin mRNA expression in tumor tissue and patient matched normal tissue;

Figure 7 illustrates the Western blot analysis using polyclonal antibody to the mammaglobin C-terminus (SEQ ID NO:14) of the conditioned medium (S) and cell lysate (C) from MDA-MB-415 breast tumor cells in the absence (-) and presence (+) of the immunizing peptide showing detection of the precursor and secreted forms of mammaglobin protein in the cell medium and cell lysate, respectively;

Figure 8 illustrates the Western blot analysis using the anti-mammaglobin polyclonal antibody of the conditioned medium (S) and cell lysate (C) from MDA-MB-415 breast tumor cells grown in the absence (-) and presence (+) of tunicamycin, which blocks glycosylation, showing the lack of detectable mammaglobin protein in the lysate or medium of cells in which N-linked glycosylation is inhibited;

Figure 9 illustrates the Western blot analysis of cell lysates from human breast tumor cells showing detection of the precursor mammaglobin protein using the anti-mammaglobin polyclonal antibody and goat anti-rabbit antibody visualized by enzyme-linked chemiluminescence;

Figure 10 illustrates the Western blot analysis using the anti-mammaglobin polyclonal antibody of fluid secretions from human breast during pregnancy and postpartum showing detection of the secreted mammaglobin protein in proliferating mammary gland;

Figure 11A illustrates in color a paraffin-fixed section of breast cancer cells from a patient specimen immunohistochemically stained using the anti-mammaglobin polyclonal antibody and goat anti-rabbit antibody tagged with horseradish peroxidase and DAB as substrate showing a brown staining of cells expressing the mammaglobin protein; and

Figure 11B illustrates in black and white a paraffin-fixed section of breast cancer cell from a patient specimen immunohistochemically stained using the anti-mammaglobin polyclonal antibody and goat anti-rabbit antibody tagged with horseradish peroxidase and DAB as substrate wherein the brown staining of cells expressing the mammaglobin protein is indicated.

Description of the Preferred Embodiments:

One aspect of the present invention is based upon the identification and sequencing of the cDNA identified as SEQ ID NO:1 which encodes a mammary-specific secretory protein, mammaglobin, identified by SEQ ID NO:2 (FIG 2). As described below, the full length mammaglobin cDNA was isolated starting from tumor cell mRNA that was reverse transcribed, amplified using the technique of PCR and subcloned into expression vectors. In addition, the protein, mammaglobin, encoded by the cDNA was identified and characterized.

Using the anonymous sequence tag previously designated DEST002, it was demonstrated that the corresponding gene product, which was up until now unknown but herein identified as mammaglobin, is particularly abundant in the breast cancer tumor cell line MDA-MB-415. To isolate the full length mammaglobin cDNA, the mRNA was reverse transcribed from this cell line and cloned using the RACE PCR technique (Edwards et al. *Nucleic Acids Research* 19:5227-32, 1991 which is incorporated by reference). This technique is based upon the strategy of ligation of single-stranded oligodeoxyribonucleotide to the 3' end of single-stranded cDNA. The method by which the mammaglobin cDNA was isolated is represented schematically in FIG 1.

The full length 503 bp cDNA sequence (SEQ ID NO:1) was deduced from the sequence information obtained from the 403 bp fragment (SEQ ID NO:5) (FIG 2) isolated by this technique along with sequence information previously obtained from the corresponding DEST sequence (DEST002, SEQ ID NO:6) (FIG 2) in our earlier study (Watson and Fleming, *supra*). Within the 503 bp cDNA is a 279 bp open reading frame which encodes a polypeptide of 93 amino acids and predicted molecular mass of 10.5kD (SEQ ID NO:2) (FIG 2). The initial methionine of this open reading frame is within a near-perfect Kozak consensus sequence (Kozak, *Cell* 22:7-8, 1980 which is incorporated by reference). The 60 bp upstream of this sequence contain no other in-frame methionines or translational stops. The 3' untranslated sequence of the cDNA constitutes 163 bp and contains a polyadenylation signal, AATAAA, 12 bp upstream of the priming site of the original DEST002 sequence. These data indicate that the full length mammaglobin cDNA has been isolated. The first 19 residues of the encoded polypeptide predict a hydrophobic

peptide signal sequence and residues 53-55 and 68-70 are consensus N-linked glycosylation sites, indicating that mammaglobin is a secreted glycoprotein.

A search for DNA sequences similar to the mammaglobin cDNA sequence in Genbank using the BLAST algorithm (Benson et al., *Nucl Acid Res* 21:2963-2965, 1993; Altschul et al, *J Mol Biol* 215:403-410, 1990 which are incorporated by reference), identified no obvious DNA sequence homologies. Thus, mammaglobin cDNA is believed to be a novel, heretofore unknown DNA sequence.

A search of other polypeptides for sequences related to mammaglobin revealed an amino acid sequence homology between mammaglobin and other polypeptides. Mammaglobin exhibited 42% amino acid identity (58% including conservative substitutions) with rat prostatic steroid binding protein (prostatein) subunit C3 (rPSC3) (FIG 3) (SEQ ID NO:7). Rat prostatic steroid binding protein is a major secretory protein in the rat ventral prostate consisting of a tetrameric protein composed of two different dimeric subunits; C3/C1 and C3/C2 (Parker et al., *Ann N Y Acad Sci* 438:115-124; Parker et al., *J Steroid Biochem* 20:67-71, 1984 which are incorporated by reference). The C1, C2, and C3 genes all encode approximately 6 kD secretory proteins and are thought to have arisen from gene duplication, but while the C1 and C2 genes show strong homology to each other, they are much less similar to the C3 gene. Correspondingly, mammaglobin shows no sequence homology with the C1 or C2 proteins.

As noted above, prostatic steroid binding protein (prostatein) is the major secretory protein in the rat ventral prostate and its expression is regulated by androgenic steroids (Parker et al, *Ann N Y Acad Sci* 438:115-24, 1984; Parker et al, *J Steroid Biochem* 20:67-71, 1984 which are incorporated by reference). Another protein, human estramustin-binding protein (hEMBP) has been reported to be expressed in human prostate, human breast cancer and human malignant melanoma. (Bjork et al, *Cancer Res* 42:1935-1942, 1982; Bjork et al, *Anticancer Res* 11:1173-82, 1991 which are incorporated by reference). Human estramustin-binding protein is immunochemically similar to rat estramustin-binding protein, which has been postulated to be identical to rat steroid-binding protein, prostatein. As noted above, the amino acid sequence of mammaglobin exhibited 42% amino acid identity and 58% homology including conservative substitutions with the C3 subunit of prostatein.

Thus it is possible that mammaglobin could be in some way related to hEMBP.

However, while both prostatein and hEMBP are detected in the prostate gland, mammaglobin mRNA is completely absent in this tissue. Hence, mammaglobin is neither the same protein nor a subunit of hEMBP and, furthermore, the sequence of hEMBP has not been determined so that it is not known whether there is even any similarity of mammaglobin with some fragment or subunit of hEMBP.

Although recent reports have demonstrated the rPSC3 promoter fused to SV40 T antigen produces both prostatic and mammary carcinomas in transgenic mice (Maroulakou et al., *Proc Nat Acad Sci U.S.* 91:11236-11240, 1994; Sandmoller et al., *Oncogene* 9:2805-2815, 1994 which are incorporated by reference), the true biological function of this protein is unknown. Furthermore, notwithstanding the hypothesized relationship of rat prostatic steroid binding protein to human EMBP, no human polypeptide or human gene corresponding to rPSC3 has been identified. Thus, mammaglobin and the cDNA encoding mammaglobin represent novel sequences heretofore unknown.

Using manual alignment with other sequences that had less significant BLAST scores with both mammaglobin and rPSC3 protein sequences, we identified other homologies with human clara cell 10kD protein (hCC10) (SEQ ID NO:8) (Peri et al, *J Clin Invest* 92:2099-2109, 1993 which is incorporated by reference) (FIG 3) and, in addition, with rabbit and mouse uteroglobin proteins (Miele et al., *Endocrine Rev* 8:474-90, 1987; Cato and Beato, *Anticancer Res* 5:65-72, 1985; Miele et al., *J Endocrinol Invest* 17:679-692, 1994 which are incorporated by reference). These homologies, depending on species, were 26% identity or 40% including conservative substitutions. In particular, a number of amino acids were perfectly conserved among all proteins, including Cys-3 and Cys-69 which are known to play a role in disulfide bond formation between uteroglobin subunits (see below). These homologies suggest that mammaglobin is a novel member of a small family of proteins that are secreted by epithelial cells (Miele et al, 1994, *supra*).

The hCC10 gene is the human homologue of rabbit and mouse uteroglobin genes (Peri et al, *J Clin Invest* 92:2099-2109, 1993 which is incorporated by reference). Uteroglobin was originally characterized as a secretory protein in rabbit uterus, but has since been found in other epithelial organs including lung, breast and

prostate. Unlike rat prostatein, uteroglobin is a homodimeric protein coupled by two disulfide linkages at the conserved residues Cys-2 and Cys-69 (Miele et al, 1994, *supra*). Although uteroglobin gene transcription is regulated by steroid hormones, the ability of the protein itself to bind progesterone or other steroid hormones is
5 controversial and again, the true biological function of this protein is unknown (Miele et al., 1994, *supra*).

Mammaglobin expression is restricted to the mammary gland. This is in contrast to the observation that rPSC3 is expressed in rat ventral prostate (Parker et al., *Ann N Y Acad Sci* 438:115-1124, 1984), and the expression of hCC10/uteroglobin
10 in numerous tissues including lung, uterus, prostate, and breast (Miele et al., 1987, *supra*; Cato and Beato, *supra*; Miele et al., 1994 *supra*). Because of the sequence homology between mammaglobin and these proteins, we determined the pattern of tissue specific expression.

The 500 bp mammaglobin mRNA was easily detected in tumor specimen
15 2410 (the tissue from which this original sequence tag was isolated) and to a much less extent in normal human breast tissue (FIG 4A). Mammaglobin mRNA could not be detected in the immortalized breast epithelial cell line B5-589. Expression of mammaglobin was also undetectable in human uterus and lung, two sites of uteroglobin expression.

20 Amplification using RT/PCR detected mammaglobin mRNA in both tumor 2410 and normal breast tissue, but not in 15 other tissues surveyed, including tissues that normally express rPSC3 and uteroglobin (lung, uterus, prostate), hormonally responsive and steroidogenic tissues (ovary, testis, placenta), and other secretory epithelial organs (colon) (FIG 4B). Therefore, the expression of
25 mammaglobin mRNA is relatively specific for mammary tissue.

Based on the studies in this report, mammaglobin is a relatively mammary-specific protein. Two other genes known to be overexpressed in breast carcinoma are erb-B and cyclin D (Jardines et al, *Pathobiology* 61:268-282, 1994; Keyomars and Pardee, *Proc Nat Acad Sci U.S.* 90:1112-1116, 1993 which is
30 incorporated by reference). Unlike the overexpression of erb-B or cyclin D, the overexpression of mammaglobin may reflect a more specific alteration of the mammary epithelial cell rather than a general increased growth potential or mitotic

rate. As such, appearance of mammaglobin gene dysregulation may have more specific import for the therapeutic vulnerability or clinical course of a tumor.

Mammaglobin expression could not be detected in normal lymph nodes or peripheral lymphocytes at the level of sensitivity afforded by a single step RT/PCR assay. This suggests that analysis of mammaglobin transcripts in peripheral lymph nodes may be useful for detecting occult breast cancer metastases, as has been suggested for other epithelial specific genes (Schoenfeld et al., *Cancer Res* 54:2986-90 which is incorporated by reference).

To demonstrate that the mammaglobin cDNA encoded a translatable protein, the cDNA clone was used in an *in vitro* translation assay. Figure 5 shows the protein product from a rabbit reticulocyte lysate programmed with the mammaglobin cDNA. An approximately 6 kD protein is generated using the mammaglobin cDNA. The apparent molecular weight is smaller than that predicted from conceptual translation of the open reading frame, but this finding is commonly observed with rabbit and human uteroglobin translation products as well.

Although we detected overexpression of mammaglobin RNA in one tumor specimen (i.e. 2410), it was not clear at what frequency this overexpression is seen in other breast carcinomas. We therefore examined a panel of fifteen, stage I primary breast carcinomas of differing histological types by Northern blot hybridization with the mammaglobin cDNA probe. Because of potential variability in expression due to environment influences (e.g. patient hormonal status), we also sought to compare tumor specimens directly with patient-matched normal breast tissues samples, although this was not possible in many cases. As shown in FIG 6, the 500 bp mammaglobin mRNA was again detected in normal breast tissue and tumor 2410. Mammaglobin was also detected in three other tumors, two of which demonstrated little or no expression in patient-matched normal tissue (BO15 and BO22). In all, 4 of 15 (27%) of tumors examined overexpressed mammaglobin mRNA. These data suggest that overexpression of mammaglobin is not unique to a single tumor specimen and is, in fact, relatively frequent among primary breast tumors. Furthermore, the fact that all tumors examined were stage I suggests that this dysregulation occurs relatively early in the progression of breast neoplasia.

Because Applicants believe mammaglobin is a secreted protein, its presence would be expected to be detectable in sera from patients whose tumors overexpress this gene product. As such, mammaglobin is likely to be as clinically useful as prostate specific antigen (PSA) and other solid tumor markers for managing patients with breast cancer (Tumor markers in diagnostic pathology, *Clin Lab Med* 10:1-250, 1990 which is incorporated by reference).

We determined the prevalence of mammaglobin as a tumor marker in the general population of breast cancer tumors by examining the expression of mammaglobin in several primary breast carcinomas. Although the number of specimens examined in this study was small, 27% of tumors evaluated overexpressed mammaglobin mRNA. This percentage is comparable to the prevalence of other genetic alterations such as erb-B amplification and p53 mutation (Slamon et al. *Sci* 244:707-712, 1989; Thor et al, *J Nat'l Cancer Inst* 84:845-855, 1992 which are incorporated by reference). Furthermore, because we have restricted our analysis to stage I tumors, overexpression of mammaglobin would actually be more prevalent than any other genetic alteration reported in this subgroup of tumors (Allerd et al, *J Nat'l Cancer Inst* 85:200-206, 1993 which is incorporated by reference).

The identification of mammaglobin as a breast cancer marker provides the basis for another aspect of the present invention, which involves methods for detecting the presence of breast cancer in a patient.

The term "detection" as used herein in the context of detection of breast neoplastic disease is intended to be a comprising aspect of the determining of the presence of breast cancer in a patient, the distinguishing of breast cancer from other diseases, the estimation of prognosis in terms of probable outcome of the disease and prospect for recovery, the monitoring of the disease status or the recurrence of the disease, the determining of a preferred therapeutic regimen for the patient and the targeting of antitumor therapy.

A method for detecting breast cancer comprises hybridizing a polynucleotide to mRNA from breast neoplasia cells. The polynucleotide comprises SEQ ID NO:1 or a derivative of SEQ ID NO:1. A derivative of a nucleotide sequence means that the derived nucleotide sequence is substantially the same as the sequence from which it is derived in that the derived nucleotide sequence has sufficient

sequence complementarity to the sequence from which it is derived to hybridize to mRNA from breast neoplasia cells under the same stringency conditions that the sequence from which it is derived hybridizes to the mRNA from breast neoplasia cells. The derived nucleotide sequence is not necessarily physically derived from the nucleotide sequence, but may be generated in any manner including for example, chemical synthesis or DNA replication or reverse transcription or transcription.

To detect the presence of mRNA encoding mammaglobin in a detection system for breast cancer, a sample is obtained from a patient. The sample can be a tissue biopsy sample or a sample of blood, plasma, serum or the like. The sample may be treated to extract the nucleic acids contained therein. The resulting nucleic acid from the sample is subjected to gel electrophoresis or other size separation techniques.

Detection involves contacting the nucleic acids and in particular the mRNA of the sample with a DNA sequence serving as a probe to form hybrid duplexes. The term "probe" refers to a structure comprised of a polynucleotide which forms a hybrid structure with a target sequence, due to complementarity of probe sequence with a sequence in the target region.

Detection of the resulting duplex is usually accomplished by the use of labeled probes. Alternatively, the probe may be unlabeled, but may be detectable by specific binding with a ligand which is labeled, either directly or indirectly. Suitable labels and methods for labeling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies, and the like.

When using the cDNA encoding mammaloglobin or a derivative thereof as a probe, high stringency conditions can be used in order to prevent false positives. When using sequences derived from mammaglobin, less stringent conditions can be used. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2d ed., 1989).

In order to increase the sensitivity of detection of mammaglobin mRNA in a sample, the technique of reverse transcription/polymerization chain reaction (RT/PCR) can be used to amplify cDNA transcribed from mRNA encoding mammaglobin. The method of RT/PCR is well known in the art (for example, see
5 Watson and Fleming, *supra*).

The RT/PCR method can be performed as follows. Total cellular RNA is isolated by, for example, the standard guanidium isothiocyanate method and the total RNA is reverse transcribed. The reverse transcription method involves synthesis of DNA on a template of RNA using a reverse transcriptase enzyme and a 3' end primer.
10 Typically, the primer contains an oligo(dT) sequence. The cDNA thus produced is then amplified using the PCR method and mammaglobin specific primers. (Belyavsky et al, *Nucl Acid Res* 17:2919-2932, 1989; Krug and Berger, *Methods in Enzymology*, Academic Press, N.Y., Vol.152, pp. 316-325, 1987 which are incorporated by reference)

15 The polymerase chain reaction method is performed using two oligonucleotide primers that are complementary to the two flanking regions of the DNA segment to be amplified. The upstream and down stream primers are typically from 20 to 30 base pairs in length and hybridize to the flanking regions for replication of the nucleotide sequence. The primers are selected to be substantially
20 complementary to the strand of cDNA being amplified. Therefore, the primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with the strand being amplified.

Polymerization from the primers is catalyzed by a DNA-polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs to produce double-
25 stranded DNA molecules. The double strands are then separated by any denaturing method including physical, chemical or enzymatic. Commonly, the method of physical denaturation is used involving heating the nucleic acid, typically to temperatures from about 80°C to 105°C for times ranging from about 1 to 10 minutes. The process is repeated for the desired number of cycles.

30 Following amplification, the PCR product is then detected by ethidium bromide staining (Sambrook, et al., 1989, *supra*).

In another embodiment of the present invention, the mammaglobin cDNA sequence or derivative thereof can be used to characterize any alteration of the mammaglobin gene (i.e. gene rearrangement, gene amplification, or gene deletion) in a specimen from a breast-cancer patient. This provides a method whereby patient
5 specimens or samples, which do not contain intact mRNA, can still be examined for changes in gene structure.

In one application of this technique, the mammaglobin cDNA sequence or derivative thereof is hybridized to patient genomic DNA that has been isolated from a patient's tumor, normal tissue, or lymphocytes and digested with one or more
10 restriction endonucleases. Using the Southern blot protocol, which is well known in the art, this assay determines whether a patient or a patient's breast tumor has a mammaglobin gene, which was deleted, rearranged, or amplified. Detection of these changes can then provide important information useful for predicting prognosis and for patient management.

In a second application of this technique, one or more pairs of oligonucleotide primers based on the mammaglobin cDNA sequence or derivative thereof could be used in the polymerase chain reaction to amplify segments of the mammaglobin gene from a patient sample. Analysis of the resulting PCR products indicate whether a particular segment of the mammaglobin gene is deleted or
15 rearranged. Such information is useful for prognosis and patient management.

Another method for detecting breast cancer comprises detecting the presence of the precursor and/or secreted forms of the mammaglobin polypeptide in a sample obtained from a patient. Any method known in the art for detecting proteins can be used. Such methods include, but are not limited to immunodiffusion,
25 immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays. (for example see *Basic and Clinical Immunology*, Sites and Terr, eds., Appleton & Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or
30 epitopes of mammaglobin and competitively displacing a labeled mammaglobin polypeptide or derivative thereof.

As used herein, the term "mammaglobin polypeptide" embraces naturally occurring mammaglobin, including nonglycosylated and glycosylated precursor forms and the glycosylated secreted form, derivatives and fragments thereof. By naturally occurring is meant a polypeptide that can be isolated from a source in nature, e.g.,
5 from normal and/or diseased organisms, and that has not been intentionally modified by man.

A derivative of mammaglobin is intended to refer to polypeptides which are comprised of a segment of at least 10 amino acids that has substantial identity to a portion of naturally occurring mammaglobin. The segment having substantial identity
10 is preferably at least about 20 amino acids, more preferably at least about 50 amino acids, and most preferably at least about 75 amino acids. Two polypeptides have substantial identity when upon optimal alignment by sequence alignment programs such as BLAST, they share at least 80 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 95 percent sequence identity, most
15 preferably 99 percent sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of
20 amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine
25 and methionine. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

A derivative mammaglobin polypeptide will preferably cross-react with an anti-mammaglobin antibody,
30 monoclonal or polyclonal, which is specific for naturally occurring mammaglobin or fragments thereof.

As used herein the terms "fragment" and "peptide" refer to a mammaglobin polypeptide having an amino acid sequence identical to the amino acid sequence deduced from a full-length mammaglobin cDNA (e.g., SEQ ID NO:1) or derivative thereof, but that has an amino-terminal and/or carboxy-terminal deletion.

5 Typically, mammaglobin fragments or peptides are at least 3 amino acids long. Preferably a mammaglobin fragment or peptide is at least 6 amino acid residues in length, more preferably about 12 amino acid residues in length, even more preferably about 25 amino acid residues in length, and most preferably 50 amino acid residues or greater.

10 Numerous competitive and non-competitive protein binding immunoassays are well known in the art. Antibodies employed in such assays may be unlabeled, for example as used in agglutination tests, or labeled for use a wide variety of assay methods. Labels that can be used include radionuclides, enzymes, fluorescers, chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors,
15 particles, dyes and the like for use in radioimmunoassay (RIA), enzyme immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassays and the like.

Polyclonal or monoclonal antibodies to a mammaglobin polypeptide comprising a B cell epitope can be made for use in immunoassays by any of a number
20 of methods known in the art. As used herein, the term "B cell epitope" refers to an antigenic determinant of a mammaglobin polypeptide. A B cell epitope could comprise 3 amino acids in a spacial conformation which is unique to the epitope. Generally, a B cell epitope consists of at least 5 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art,
25 and include, for example, x-ray crystallography and 2 dimensional nuclear magnetic resonance.

One approach for preparing antibodies to a protein is the selection and preparation of an amino acid sequence of all or part of the protein, chemically synthesizing the sequence and injecting it into an appropriate animal, usually a rabbit
30 or a mouse.

Methods for preparation of a mammaglobin polypeptide include, but are not limited to chemical synthesis, recombinant DNA techniques or isolation from

biological samples. Chemical synthesis of a peptide comprising an epitope can be performed, for example, by the classical Merrifield method of solid phase peptide synthesis (Merrifield, *J Am Chem Soc* 85:2149, 1963 which is incorporated by reference) or the Fmoc strategy on a Rapid Automated Multiple Peptide Synthesis system (DuPont Company, Wilmington, DE) (Caprino and Han, *J Org Chem* 37:3404, 1972 which is incorporated by reference).

Polyclonal antibodies can be prepared by immunizing rabbits by injecting antigen into the popliteal lymph nodes followed by subsequent boosts at two week intervals with intraperitoneal injection of antigen. The animals are bled and sera assayed against purified mammaglobin protein, usually by ELISA.

Monoclonal antibodies can be prepared after the method of Milstein and Kohler by fusing splenocytes from immunized mice with continuously replicating tumor cells such as myeloma or lymphoma cells. (Milstein and Kohler *Nature* 256:495-497, 1975; Gutfre and Milstein, *Methods in Enzymology: Immunochemical Techniques* 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). The hybridoma cells so formed are then cloned by limiting dilution methods and supernates assayed for antibody production by ELISA or RIA.

Thus prepared polyclonal or monoclonal antibodies to mammaglobin may be used to isolate and purify precursor and secreted forms of mammaglobin from cells expressing mammaglobin. For example, as shown below, a polyclonal antibody generated against the 16 C-terminal amino acids predicted from mammaglobin cDNA (Glu-Val-Phe-Met-Gln-Leu-Ile-Tyr-Asp-Ser-Ser-Leu-Cys-Asp-Leu-Phe, SEQ ID NO:14) binds to precursor and secreted forms of mammaglobin, as well as to mammaglobin that has been synthesized in an *in vitro* translation system. Isolation of mammaglobin using an anti-mammaglobin antibody may be accomplished using procedures well-known in the art, such as affinity chromatography.

The unique ability of antibodies to recognize and specifically bind to target antigens expressed by a tumor cell provides an approach for the treatment of cancer. (For review see LoBuglio and Saleh, *Am J Med Sci* 304:214-224, 1992; Bagshawe, *Adv Pharmacol* 24:99-121, 1993 which are incorporated by reference). Thus, another aspect of the present invention provides for a method for preventing the onset and

treating breast cancer in an animal based upon the use of antibodies to mammaglobin, which has been discovered to be overexpressed by breast cancer cells.

Specific antibodies to mammaglobin, either polyclonal or monoclonal, are produced by any method known in the art. For example, murine or human

5 monoclonal antibodies can be produced by hybridoma technology. Alternatively, mammaglobin, or an immunologically active derivative or fragment thereof, or an anti-idiotypic antibody, or fragment thereof, can be administered to an animal to elicit B cell production of antibodies capable of recognizing the mammaglobin-expressing cells.

10 The antibodies so produced or fragments thereof are labeled with one or more oncolytic substances such as radionuclides, toxins, or cytotoxic drugs and administered to a patient suspected of having breast cancer. The binding of the labeled antibody to the mammaglobin being overexpressed by the breast cancer cell will cause the death of the cancer cell.

15 Any of a variety of oncolytic substances known in the art can be used to produce such labeled antibodies. For example, immunotoxins can be made by coupling plant and bacterial toxins to antibodies. Such toxins include, for example, ricin, diphtheria toxin and *Pseudomonas* exotoxin A. Drug-antibody conjugates can also be made in which chemotherapeutic agents are linked to the antibody.

20 Chemotherapeutic agents suitable for such use include, for example, tomosifen, doxorubicin, methotrexate, chlorambucil, *Vinca* alkaloids, and mitomycin. In addition, radioimmunoconjugates can be made in which a radionuclide is stably linked to the antibody. Radionuclides suitable for making radioimmunoconjugates include, for example, β -emitters such as ^{131}I , ^{188}Re , ^{186}Re , ^{67}Cu , ^{90}Y and ^{47}Sc ; α -
25 emitters such as ^{211}At , ^{212}Bi and ^{212}Pb ; auger electron emitters such as ^{125}I and ^{77}Br ; and fissionable nuclides such as ^{10}B .

The finding that a significant percentage of breast tumors express mammaglobin protein is the basis for another aspect of the invention, which involves the activation of mammaglobin-specific B and/or T cell lymphocytes (T_C) with
30 mammaglobin antigens. Accordingly, the invention provides mammaglobin B cell antigens and T_C cell antigens; vaccines comprising at least one B cell mammaglobin antigen and/or at least one T_C mammaglobin antigen for inducing antibody- and/or

cell-mediated immune responses against mammaglobin-expressing tumors, and methods for treating a breast cancer patient with a mammaglobin-expressing tumor.

One method according to the invention comprises administering to the patient activated mammaglobin-specific lymphocytes. Another method comprises

5 administering to the patient a mammaglobin-specific vaccine.

As used herein, "mammaglobin antigen" includes naturally occurring mammaglobin polypeptides, derivatives, and fragments thereof which contain a B cell or T_C cell epitope recognized by mammaglobin-specific B cells or T_C cells.

A mammaglobin B cell antigen comprises a mammaglobin-specific B cell
10 epitope and a T_H cell epitope. The term "B-cell epitope" refers to any antigen, hapten, epitope or antigenic determinant which is recognized by anti-mammaglobin immunoglobulin receptors on B cells and is capable of eliciting the production of antibodies with appropriate help from T_H cells when administered to an animal. The B cell epitope comprises an amino acid sequence of at least 4 amino acids.

15 Preferably, the B cell epitope is between at least 6 and 25 amino acids in length and more preferably is between about 15 and 22 amino acids in length. The comprising amino acid sequence of the B cell epitope may be identical or substantially identical to a continuous amino acid sequence in a fragment of naturally occurring mammaglobin. Alternatively, the comprising amino acid sequence of a B cell epitope
20 is identical to or substantially identical to a discontinuous amino acid sequence representing an assembled topographic determinant of mammaglobin.

The term "T_H cell epitope" refers to any antigenic determinant recognized by T helper cells through association with MHC class II molecules. The activation of T helper cells induces differentiation of resting mammaglobin-specific B cells into
25 higher affinity IgG-secreting cells, i.e., induces a secondary antibody response. The preparation and use of immunogenic peptides containing B and T_H cell determinants to produce higher titres of specific antibody-producing B cells through T cell help is known in the art, see, e.g., Cheronis et al., U.S. Patent No. 5,573,916, Denton, et al., *Cancer Letters* 70:143-150 (1993), Borrás-Cuesta et al., *Eur. J. Immunol.* 17, 1213-
30 1215 (1987), and Good et al., *Science* 235:1059-1062 (1987), each of which is incorporated herein by reference. The T_H cell epitope may comprise an amino acid sequence from mammaglobin or a heterologous protein. For example, Denton et al.

describe the induction of antibody responses to mucins, which are complex glycoproteins expressed in secretory epithelia and associated with breast and other carcinomas, in mice immunized with a synthetic peptide containing a B cell determinant region from the core of MUC-1 mucin linked to sequence 111-120 of influenza haemagglutinin A/X-31, a known helper T cell-determinant. The T_H cell epitope comprises an amino acid sequence of between about 6 to about 20 amino acid residues, preferably between about 8 residues and 18 residues, even more preferably between 9 residues and 15 residues.

A mammaglobin T_C cell antigen comprises a T_C cell epitope and a MHC class I agretope. The term " T_C cell epitope" means any antigen, epitope or antigenic determinant which is recognized by mammaglobin-specific T_C cells when presented by a MHC class I molecule on the surface of an antigen presenting cell. The term "MHC class I agretope" refers to any amino acid sequence recognized by a MHC class I molecule that allows the mammaglobin antigen to be presented to a mammaglobin-specific T_C cell by the MHC class I molecule on an antigen presenting cell (APC). The T_C cell epitope and MHC class I agretope are contained within an amino acid sequence of between about 6 to about 11 amino acids that is identical or substantially identical to the amino acid sequence of a fragment of naturally occurring mammaglobin. Preferably, the sequence is 8 or 9 amino acids in length.

Methods for identifying B and T_C cell epitopes for a protein antigen are known in the art. For example, the capacity of isolated mammaglobin-specific B cells or mammaglobin-specific T_C cells to respond to overlapping synthetic peptides spanning secreted mammaglobin may be determined using standard immunobiology techniques. Those peptides identified as antigenic may then be modified one or a few amino acids at a time to optimize their ability to stimulate mammaglobin-specific B or T cells.

B cell epitopes can also be mapped using commercially available epitope mapping kits which involve the screening of random peptides bound at the C terminus to polyethylene multipin supports, e.g., Cambridge Research Biochemicals.

Alternatively, the predicted mammaglobin amino acid sequence may be searched for sequences that conform to known binding motifs of MHC class I or MHC class II molecules. See e.g., Hill et al., *Nature* 360:434 (1992), Pamer et al.,

Nature 360:852 (1992) and Hammer et al., *J. Exp. Med.* 176:1007 (1992), and Falk et al., *Nature* 351:290-296 (1991), each of which is herein incorporated by reference. For example, antigenic peptides recognizable by breast tumor-specific CTLs may be identified by searching the mammaglobin amino acid sequence for HLA-A2-binding peptides as described by Peoples et al., *Proc. Natl. Acad. Sci.* 92:432-436 (1995), which is incorporated herein by reference. The choice of HLA-A2 as the antigen presenting molecule is appropriate where the patient expresses HLA-A2 (approximately 50% of Caucasians express HLA-A2). The predicted HLA-A2 binding peptides can be synthesized and tested for antigenicity by loading the synthetic peptides onto the T2 cell line, a human T-cell/B-cell fusion product containing a defect in antigen presentation such that HLA-A2 molecules on the surface of T2 cells can be effectively loaded with exogenous HLA-A2 binding peptides (Henderson, et al, *Science* 255:1264-1266 (1992) incorporated herein by reference). A standard cytotoxicity assay is then carried out which comprises incubating the peptide-loaded T2 cells with breast-specific CTLs derived from tumor infiltrating lymphocytes (TILs) isolated from a mammaglobin-expressing breast tumor, e.g., see Peoples et al., pages 432-433 and Toso et al., *Cancer Research* 56:16-20 (1996), herein incorporated by reference.

Antigenic mammaglobin peptides containing T_C cell epitopes may also be identified by acid-eluting endogenous peptides presented by HLA class I molecules on the tumor cell surface. (See, e.g., Peoples et al., *supra*, p. 433). The eluted peptides may be separated by any number of techniques known in the art, including HPLC fractionation. The different peptide fractions are loaded onto T2 cells and the loaded T2 cells are incubated with breast-tumor specific CTLs to determine which peptides are recognized by the CTLs using standard immunobiology techniques.

One use of a mammaglobin antigen according to the invention is in adoptive immunotherapy. This therapy involves in vitro activation and expansion by a mammaglobin antigen of anti-mammaglobin antibody-producing B cells and/or mammaglobin-specific cytotoxic T lymphocytes (CTLs) isolated from a patient with a mammaglobin-expressing tumor. The method may also be practiced with a composition comprising both mammaglobin B cell and T_C cell antigens. The

activated lymphocytes are then introduced back into the patient for adoptive immunotherapy.

A mammaglobin antigen according to the invention is also useful as a component of a mammaglobin-specific vaccine. The vaccine comprises an immunogenically-stimulatory amount of a mammaglobin antigen. As used herein, an immunostimulatory amount refers to that amount of antigen that is able to stimulate the desired immune response in the recipient for the amelioration, or treatment, of breast cancer. This amount may be determined empirically by standard procedures, well known to those of ordinary skill in the art, without undue experimentation.

The antigen may be provided in any one of a number of vaccine formulations which are designed to induce the desired type of immune response, e.g., antibody and/or cell mediated. Such formulations are known in the art. See, e.g., A. Lanzavecchia, *Science* 260:937-944 (1993) and U.S. Patent No. 5,585,103 to Raychandhuri, each of which is incorporated herein by reference. Examples of vaccine formulations used to stimulate immune responses include pharmaceutically acceptable adjuvants such as aluminum salts; emulsions of squalene or squalane and muramyl dipeptide; liposomes; and a composition comprising a stabilizing detergent, a micelle-forming agent, and a biodegradable and biocompatible oil (Raychandhuri, *supra*).

A mammaglobin-specific vaccine may also comprise a carrier cell loaded with a mammaglobin antigen. Preferably, the carrier cell is prepared from autologous professional antigen presenting cells (APC) such as macrophages, dendritic cells, or activated B or T lymphocytes. See e.g., Lanzavecchia, *supra*, p. 937. Professional APCs express a ligand, B7, that binds to CD28 or CTLA4 on T cells to deliver an antigen- nonspecific costimulatory signal known as Signal 2 which prevents T cell anergy or inactivation. Thus, the vaccine may also comprise interleukin-2 or another costimulatory signal to counteract anergy induction. (Lanzavecchia, *supra*, p. 938.)

Another formulation of a mammaglobin-specific vaccine comprises a recombinant vector containing a nucleotide sequence encoding for expression a mammaglobin antigen. The use of infectious agents to stimulate cytotoxic T lymphocytes is known in the art. (Raychaudhuri, *supra*.) Chimeric vectors have been described using vaccinia, polio, adeno-and retro-viruses, as well as bacteria such as

Listeria and BCG. For example, a canarypox virus vector, ALVAC, has been shown to elicit strong cellular immune responses against encoded heterologous gene products (Taylor et al, *Virology* 187:321-328 (1991), incorporated herein by reference). In addition, a recombinant ALVAC expressing the MZ2-E human melanoma rejection antigen encoded by the MAGE-1 gene is able to stimulate *in vitro* MAGE-1 CTL activities in a TIL population derived from a breast tumor expressing MAGE-1 mRNA (Toso et al, *supra*). In another approach described in U.S. Patent No. 5593972 to Weiner et al (herein incorporated by reference), a recombinant expression vector encoding an antigen of an immunogenic protein to be targeted is directly administered to an individual either *in vivo*, e.g., to muscle cells, or to the cells of an individual *ex vivo* along with an agent that facilitates uptake of the DNA into the cells.

Those skilled in the art may readily determine how to formulate a vaccine suitable for achieving the desired immune response. For example, for inducing *in vivo* production of anti-mammaglobin antibodies, a mammaglobin-specific vaccine comprises at least one mammaglobin B cell antigen comprising a B cell epitope and a T_H cell epitope. The T_H cell epitope is preferably matched with the appropriate MHC class II haplotype of the intended vaccine recipient. Alternatively, a T_H cell epitope could be used that is known to be recognized universally by humans regardless of HLA type such as the "universal" T cell epitope from tetanus toxoid (Panina-Bordignon et al. *Eur. J. Immunol.* 19:2237 (1989), herein incorporated by reference). Preferably, the vaccine comprises a plurality of mammaglobin B cell antigens with T_H epitopes recognized by MHC Class II molecules of different HLA types.

Another embodiment of a mammaglobin-specific vaccine induces a cell-mediated response and comprises at least one mammaglobin T_C antigen capable of activating mammaglobin-specific T_C cells. Preferably, the vaccine comprises several T_C cell antigens.

A mammaglobin-specific vaccine may also be formulated to induce both antibody and cell-mediated responses. This embodiment comprises both mammaglobin B cell and T_C cell antigens.

A patient with a mammaglobin-expressing tumor may be treated by administering to the patient an immunostimulatory amount of a mammaglobin-

specific vaccine according to the present invention. Administration of the vaccine may be by any known or standard technique. These include, but are not limited to intravenous, intraperitoneal, intramuscular, subcutaneous, or intramammary injection.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

In the examples below, cell lines were obtained from American Type Culture Collection and grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Tissue biopsy specimens were obtained from the Human Cooperative Tissue Network (LiVolsi et al, *Cancer* 71:1391-1394, 1993 which is incorporated by reference).

Example 1

This example illustrates the isolation of mammaglobin cDNA.

Total cellular RNA from the cell line MDA-MB415 was isolated using the standard guanidinium isothiocyanate method. (Belyavsky et al, *supra*). This RNA was used in the RACE PCR procedure employing the Amplifinder kit (Clontech) and following the manufacturer's protocol.

The synthesis of first strand cDNA was performed in a standard reaction containing 1 µg RNA, 10 µM specific mammaglobin primer D2R (5'-ATA AGA AAG AGA AGG TGT GG-3')(SEQ ID NO:4), 4µl of 5X RT buffer (250 mM TrisCl pH8.3, 375mM KCl, 15mM MgCl₂), 2 µl of 100mM DTT, 1 µl of 10 mM dNTPs and 200 units of Superscript™ II reverse transcriptase (Gibco/BRL) in a reaction volume 20 µl. The reaction proceeded for 1 hour at 45°C and was terminated by incubating at 95°C for 5 minutes. RNA was hydrolyzed with 400 µM NaOH at 65°C for 30 minutes and neutralized with 400 µM acetic acid.

The reaction mixture was then added to 3 volumes of 6M NaI and 10 µl of treated glass beads. Beads were washed three times with 80% EtOH and nucleic acid was eluted from the beads in 45 µl of water. Nucleic acid was then precipitated and

26

resuspended in 10 µl of water. The purified first strand cDNA was ligated to the manufacturer's provided anchor oligonucleotide (SEQ ID NO:9, 5'-CAC GAA TTC ACT ATC GAT TCT GGA ACC TTC AGA GG-3'), using T4 RNA ligase at 27° for 20 hours. One tenth of a ligation reaction was used for PCR amplification in a 50 µl
5 reaction containing 1 µM manufacturer's anchor primer (SEQ ID NO:10, 5'-CTG GTT CGG CCC ACC TCT GAA GGT TCC AGA ATC GAT AG-3'), 1 µM mammaglobin specific primer D2Rb (SEQ ID NO:11, 5'-AAT CCG TAG TTG GTT TCT CAC C-3'), 200 µM dNTPs, 5 units of Vent™ DNA polymerase, and 1X polymerase buffer (10mM KCl, 20 mM TrisCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄,
10 0.1% Triton X-100). The reaction was incubated at 94° for 2 minutes and then 94° for 45 seconds, 50° for 1 minute, and 72° for 90 seconds for a total of 40 times.

The two downstream mammaglobin-specific nested oligonucleotides were D2R (SEQ ID NO:4) and D2Rb (SEQ ID NO:11). An upstream mammaglobin-specific control oligonucleotide was also used as per the manufacturer's
15 recommendations, D2F (5'-CTT TCT GCA AGA CCT TTG GC-3') (SEQ ID NO:12). All PCR amplifications were performed with Vent DNA polymerase (New England Biolabs). The amplified RACE product was digested with EcoRI and ligated into the EcoRI and SmaI sites of the plasmid vector pGEM7Z (Promega, Madison, WI).

All sequencing was performed using the Taq DNA polymerase thermal
20 cycle sequencing kit as per the manufacture's protocol (Promega) Briefly the procedure used is as follows.

Ten pmol of sequence specific oligonucleotide was end labeled with 10 pmol of ³²P-γ ATP (3,000 Ci/mmol and 10 mCi/ml) using T4 polynucleotide kinase in a 10 µl reaction for 30 minutes at 37°C. A polymerization reaction containing 100 ng
25 of plasmid template, 1.5 pmol of labeled sequencing primer, and 5 units of sequencing grade Taq polymerase was created in 17 µl of the manufacturer's provided sequencing buffer. This reaction was aliquoted to a set of four reaction tubes containing manufacturer's provided mix of deoxynucleotides and either dideoxy-A, C, G, or T. The set of four tubes were incubated at 95°C for 2 minutes and then, 94°C
30 for 45 seconds, 45°C for 30 seconds, and 72°C for 1 minute for 30 times. After reactions were completed, 3 µl of 80% formamide/bromphenol blue dye was added to each tube. Samples were heated to 70°C for 2 minutes and loaded on a 6%

acrylamide/7.5 M urea sequencing gel and run for 2-4 hours and 60 W constant power. The gel was dried and then exposed to Kodak XAR5 Xray film for 2 to 24 hours.

The sequence thus obtained was a 403 bp fragment (SEQ ID NO:5) as shown in FIG 2, solid bar. In earlier work the DEST002 Tag sequence was isolated (Watson and Fleming, *supra*). This sequence was a 206 bp fragment (SEQ ID NO:6) as shown in FIG 2, open bar. Combining the information from these two sequences allowed the full-length 503 bp cDNA of mammaglobin to be deduced. (FIG 2).

10 Example 2

This example demonstrates that mammaglobin expression is restricted to mammary gland tumor cells and, to a lesser extent, normal mammary gland cells.

Total cellular RNA samples were isolated using the standard guanidinium isothiocyanate method and treated with RNase-free DNase (Promega). For RT/PCR
15 analysis, 1 µg of indicated total RNA was reverse transcribed with oligo dT₂₁ (SEQ ID NO:13) and Superscript II reverse transcriptase (Gibco/BRL) according to the manufacture's protocol.

Two hundred ng of oligo dT₂₁ (SEQ ID NO:13) and 1 µg of total RNA were incubated at 65°C for 5 minutes in a 10 µl volume. Sample was chilled on ice
20 and added to it were 4µl of 5X RT buffer (250 mM TrisCl pH8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl of 100mM DTT, 1 µl of 10mM dNTPs and 200 units of Superscript™ II reverse transcriptase (Gibco/BRL). The reaction proceeded for 1 hour at 45°C and was terminated by incubating at 95°C for 5 minutes.

One tenth of each RT reaction was subject to PCR analysis using the
25 mammaglobin specific primers D2R (5'-ATA AGA AAG AGA AGG TGT GG-3') (SEQ ID NO:4) and d2102 (5'-CAG CGG CTT CCT TGA TCC TTG-3') (SEQ ID NO:3) and standard reaction conditions for 40 cycles at 94° x 30 sec./55° x 1 min./72° x 1 min.

For Northern analysis, 20 µg of total RNA was analyzed as previously
30 described (Watson and Fleming, *supra*) using the full length mammaglobin cDNA probe. Integrity and equal loading of each RNA sample was assessed by ethidium bromide staining.

As shown in FIG 4A, the 500 bp mammaglobin message is easily detected in tumor specimen 2410 (the tissue from which this original DEST was isolated) and to a much lesser extent in normal human breast tissue but not in the immortalized breast epithelial cell line B5-589, or in human lung, placenta, uterus and ovary (FIG 4A). Following amplification using RT/PCR analysis, mammaglobin expression was still not detected in 15 tissues surveyed (FIG 4B). Detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message (FIG 4B) and EGF receptor message (data not shown) in these reactions demonstrated that absence of expression was not due to degraded RNA or other trivial explanations. Thus the expression of mammaglobin mRNA is relatively specific for mammary tissue.

Example 3

This example demonstrates that the mammaglobin cDNA encodes a translatable nucleotide sequence which results in protein product of appropriately predicted molecular mass. *In vitro* translations were performed using the TNTTM rabbit reticulocyte translation kit with T7 RNA polymerase (Promega) and ³⁵S-Methionine (>1000 Ci/mmol; 10 mCi/ml, Amersham) according to the manufacturer's protocol.

To 25 μ l of TNTTM rabbit reticulocyte lysate was added 2 μ l of manufacturer's prepared reaction buffer, T7 RNA polymerase, 20 μ M amino acid mixture minus methionine, 40 μ Ci ³⁵S-methionine (1,000 Ci/mmol and 10 mCi/ml), 40 units ribonuclease inhibitor, 1 μ g of mammaglobin/pGEM7 plasmid, and sufficient DEPC treated water to create a final reaction volume of 50 μ l. This reaction was incubated at 30°C for 60 minutes. 5 μ l of this reaction was removed into 20 μ l of SDS gel buffer, boiled for 2 minutes, and loaded on a 17.5% SDS-polyacrylamide gel.

Rabbit reticulocyte lysate programmed with mammaglobin cDNA produced a 6kD protein while that programmed with no cDNA did not produce any protein product.

Example 4

This example illustrates the prevalence of overexpression of mammaglobin in primary breast carcinoma.

To determine the frequency of mammaglobin overexpression in breast carcinomas, we examined a panel of fifteen, stage I primary breast carcinomas of differing histological types using Northern blot hybridization with the mammaglobin cDNA probe. Patient-matched normal breast tissues samples were also compared in tissues from two patients (FIG 6). The 500 bp mammaglobin mRNA was detected in normal breast tissue and tumor 2410 and in three other tumors, two of which when tested demonstrated little or no expression in patient-matched normal tissue (BO15 v. BO16; BO22 v. BO23) (FIG 6). In all, 4 of 15 (27%) of tumors examined overexpressed mammaglobin mRNA.

These data indicate that overexpression of mammaglobin is not unique to a single tumor specimen and is, in fact, relatively frequent among primary breast tumors. Furthermore, the fact that all tumors examined were stage I suggests that this dysregulation occurs relatively early in the progression of breast neoplasia.

EXAMPLE 5

The following example illustrates the detection of the mammaglobin protein using an anti-mammaglobin polyclonal antibody.

The anti-mammaglobin polyclonal antibody was prepared by coupling a peptide corresponding to the 16 C-terminal amino acids predicted from mammaglobin cDNA (Glu-Val-Phe-Met-Gln-Leu-Ile-Tyr-Asp-Ser-Ser-Leu-Cys-Asp-Leu-Phe, SEQ ID NO:14) to Keyhole Lymphet Hemocyanin and injecting into rabbits with Freund's adjuvant. The inoculated rabbits were boosted at three week intervals and on week 12, the rabbits were bled and the sera was assayed for its ability to detect mammaglobin in serum-free conditioned medium from cultures of the breast tumor cell lines MDA-MB-415 and MCF-7. MDA-MB-415 had been identified earlier as a cell line that overexpresses the mammaglobin message and MCF-7 had been identified as a cell line that produces no detectable mammaglobin mRNA.

The conditioned media was harvested from a 24 hr. culture and resolved on a 12% SDS acrylamide gel under reducing conditions (i.e., the sample was boiled

in buffer containing dithiothreitol (DTT) and 2-mercaptoethanol (BME) to reduce disulfide bonds), blotted onto a Nytran filter, and analyzed by standard Western blot protocols using the above described antibody to the C-terminal peptide as the primary antibody in this assay. After primary antibody binding, the blot was washed and
5 secondary antibody (goat anti-rabbit) was added. Mammaglobin-antibody complexes were visualized by enzyme-linked chemiluminescence (ECL Western Blotting Detecting Reagent, Amersham, Arlington Heights, IL).

The anti-mammaglobin polyclonal antibody detected a band with an apparent molecular weight of approximately 21 kD in the conditioned media of the
10 MDA-MB-415 cell culture (data not shown). No bands were detected in the conditioned medium of the MCF-7 cell culture (data not shown). Thus, consistent with the mRNA data, MDA-MB-415 cells secrete mammaglobin protein but MCF-7 cells do not.

The apparent molecular weight of the mammaglobin secreted into the
15 MDA-MB-415 culture media is greater than the 10.5 kDa molecular weight calculated from the predicted amino acid sequence of SEQ ID NO:2. Since almost all secreted proteins are glycosylated, the cytosol of MDA-MB-415 cells was analyzed with the anti-mammaglobin polyclonal antibody to see if any precursor forms of secreted mammaglobin could be detected.

20 MDA-MG-415 cells were grown for 24 hours in serum-free media, the culture media was collected, spun, and the resulting supernatant was collected. The attached cells were washed with phosphate buffered saline (PBS) and lysed with 1X Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris, pH 6.8, 0.001% Bromophenol Blue). The lysis mixture was boiled for 5 minutes and then
25 spun at 10,000 g for 5 minutes to pellet the cell debris. The cell lysate was transferred to a new tube and used for Western blot analysis as described below.

The culture supernatant and cell lysate were run on a 12% SDS acrylamide gel under reducing conditions (i.e., samples boiled in buffer containing DTT and BME) and blotted onto a PVDF membrane using standard techniques. The blot was
30 probed with the polyclonal antibody to the C-terminal peptide in the presence and absence of the competing peptide used to generate the antibody.

Visualization of mammaglobin-antibody complexes were as discussed above. As seen in Figure 7, in the absence of competing peptide (-), the conditioned media (S) has the 21 kD band representative of the secreted mammaglobin protein. The cell lysate (C) showed a prominent band at approximately 14 kD, and several
5 higher molecular weight bands, including one at approximately 21 kD. When the Western blot is performed in the presence of the competing peptide (+), the secreted form and intracellular forms of mammaglobin are not visualized, indicating that these proteins contain the peptide to which the antibody was synthesized.

The 14 kD band detected only in the cell lysate likely represents a
10 precursor, or unprocessed, form of mammaglobin. Since the predicted amino acid sequence for mammaglobin has the consensus N-glycosylation site, Asn-X-Thr, located at residues 53-55 and at residues 68-70 of SEQ ID NO:2, the observed, secreted 21 kD form likely represents some glycosylated form of the protein.

This hypothesis was tested by culturing MDA-MB-415 cells in the
15 presence and absence of tunicamycin, a drug that blocks N-linked glycosylation of eukaryotic proteins. Tunicamycin was added to one of two identical cultures at 1 ug/ml and both cultures were incubated overnight for more hours. The culture media and cell lysate from the treated and control cultures were prepared and analyzed by Western blot analysis as described above.

20 As shown in FIG 8, media from cultures (S) treated with tunicamycin (+) lack detectable levels of secreted mammaglobin, suggesting that secreted mammaglobin is glycosylated. Surprisingly, the cell cytosol form of mammaglobin (14 kD) was also not detectable in lysates of MDA-MB-415 cells treated with tunicamycin (far right lane). We hypothesize that blocking early glycosylation events
25 with tunicamycin leads to instability and degradation of precursor forms of mammaglobin, thus explaining the lack of detectable 14 kD protein in the cytosol of tunicamycin-treated cells.

The polyclonal antibody to the C-terminal peptide of mammaglobin has also detected the 14 kDa precursor form of mammaglobin in cell lysates from primary
30 human breast tumor specimens. As seen in FIG 9, the precursor form of mammaglobin is present in tumor specimen BO23, but is undetectable in a normal breast tissue sample from the same patient (B022). Interestingly, some tumor samples

that express the mammaglobin transcript (i.e., 087R, 014, 75A and 2410) do not contain detectable levels of mammaglobin protein as assayed by Western blot analysis. One hypothesis consistent with these data is that mammaglobin expression is differentially regulated at the levels of transcription and translation and that this differential regulation is determined the developmental stage of the tumor.

The anti-mammaglobin polyclonal antibody has also been used to look for secreted mammaglobin in breast secretions from proliferating mammary gland. Colostrum or mature milk fluid (500 ul samples) was collected by manual expression from a pregnant woman during the first and third trimester, at birth, and at day 3, 14, and 21 post-partum. The samples were diluted with an equal volume of 2X laemmli sample buffer (4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris, pH 6.8, 0.002% Bromophenol Blue). The diluted samples were boiled for 5 min. and then spun at 10,000 g for 5 min. at 4°C to pellet cell debris. The denatured samples were transferred to a new tube and stored at -20°C prior to Western blot analysis as described above.

As shown in FIG 10, the antibody detected the 21 kD secreted mammaglobin in breast secretions sampled during pregnancy, a period of high proliferation of breast epithelial cells. However, at the onset of lactation, a stage of breast epithelial differentiation, mammaglobin levels decreased significantly by 3 days post partum and was no longer observed at 14 days post-partum. These results indicate that secreted mammaglobin is associated with proliferating breast epithelial cells, an observation consistent with the detection of secreted mammaglobin in human breast cancer.

Reactivity with the antibody to the mammaglobin peptide has also been shown for breast tumor cells by immunohistochemical staining of paraffin-fixed sections of a breast cancer patient specimen (FIG 11). The immunohistochemical staining was performed using the antibody to the mammaglobin peptide as the primary antibody and then detecting the mammaglobin-antibody complex using goat anti-rabbit antibody tagged with horseradish peroxidase and 3, 3' diamino benzene tetrahydrochloride (DAB) as substrate. Cells expressing the mammaglobin protein showed a brown staining.

From these results, it is believed that the mammaglobin protein is synthesized as a precursor protein and post-translational modifications such as N-linked glycosylation increase its apparent molecular weight prior to secretion; that the stability of precursor forms of mammaglobin is dependent on N-linked glycosylation; and that mammaglobin protein is secreted by proliferating breast tumor cells. The detection of a mammaglobin protein is applicable in cancer diagnostics using the mammaglobin protein as a breast tumor marker, in assessing breast tumor relapse, in monitoring autologous bone marrow/stem cell transplants for contaminating tumor cells, and in targeting breast tumor cells for therapeutic intervention via antibody-mediated complexes. A purified and isolated mammaglobin polypeptide is useful for generating antibodies against breast tumors and in the development of other tumor-specific immunotherapy regimens.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

What is Claimed is:

1. A mammaglobin-specific vaccine comprising an immunogenically-effective amount of at least one mammaglobin antigen.
2. The vaccine of Claim 1, wherein said mammaglobin antigen is loaded onto a carrier cell.
3. The vaccine of Claim 1, further comprising a pharmaceutically acceptable adjuvant.
4. The vaccine of Claim 1, comprising a mixture of mammaglobin B cell antigens and mammaglobin T_c cell antigens.
5. A mammaglobin-specific vaccine comprising a recombinant vector which comprises a nucleotide sequence encoding for expression a mammaglobin B cell antigen and/or a mammaglobin T_c cell antigen.
6. A method for treating a patient with a mammaglobin-expressing tumor which comprises administering to the patient a mammaglobin-specific vaccine comprising an immunogenically-effective amount of a mammaglobin antigen.
7. The method of Claim 6, wherein said antigen is loaded onto a carrier cell.
8. The method of Claim 5, wherein said vaccine also comprises a pharmaceutically acceptable adjuvant.
9. The method of Claim 5, wherein said mammaglobin antigen comprises an amino acid sequence which is encoded for expression by a nucleotide sequence in a recombinant vector.
10. A method for treating a patient with a mammaglobin-expressing breast tumor which comprises isolating mammaglobin-specific lymphocytes from the tumor, activating the lymphocytes with at least one mammaglobin antigen, and administering the activated lymphocytes to the patient.
11. An isolated and purified polypeptide comprising at least one mammaglobin antigen which comprises at least six contiguous amino acids of SEQ ID NO:2, wherein said mammaglobin antigen is recognized by B cells and/or T_c cells specific for a naturally occurring, secreted mammaglobin polypeptide which is glycosylated and consists of amino acids 20-93 of SEQ ID NO:2.
12. The isolated and purified polypeptide of Claim 11, wherein the mammaglobin antigen comprises at least 12 contiguous amino acids of SEQ ID NO:2.

13. The isolated and purified polypeptide of Claim 12, wherein the mammaglobin antigen comprises at least 25 contiguous amino acids of SEQ ID NO:2.

14. The isolated and purified polypeptide of Claim 13, wherein the mammaglobin antigen is glycosylated and consists of amino acids 20-93 of SEQ ID NO:2.

15. The isolated and purified polypeptide of Claim 13, wherein the mammaglobin antigen is glycosylated and consists of SEQ ID NO:2.

16. The isolated and purified polypeptide of Claim 11, wherein the mammaglobin antigen is a mammaglobin B cell antigen which induces *in vitro* activation and expansion of B cells from a breast cancer patient.

17. The isolated and purified polypeptide of Claim 16, wherein the mammaglobin B cell antigen comprises a T_H cell epitope from a heterologous protein.

18. The isolated and purified polypeptide of Claim 11, wherein the mammaglobin antigen is a mammaglobin T_C cell antigen which induces *in vitro* activation and expansion of T_C cells from a breast cancer patient.

19. The isolated and purified polypeptide of Claim 18, wherein the mammaglobin T_C cell antigen comprises 8 or 9 contiguous amino acids of SEQ ID NO:2.

20. The isolated and purified polypeptide of Claim 11, which comprises a mammaglobin B cell antigen and a mammaglobin T_C cell antigen.

AMENDED CLAIMS

[received by the International Bureau on 27 January 1999 (27.01.99);
original claim 1 amended; remaining claims unchanged (1 page)]

1. A mammaglobin-specific vaccine comprising an immunogenically-effective amount of at least one mammaglobin antigen which comprises at least six contiguous amino acids of SEQ ID NO:2.
2. The vaccine of Claim 1, wherein said mammaglobin antigen is loaded onto a carrier cell.
3. The vaccine of Claim 1, further comprising a pharmaceutically acceptable adjuvant.
4. The vaccine of Claim 1, comprising a mixture of mammaglobin B cell antigens and mammaglobin T_c cell antigens.
5. A mammaglobin-specific vaccine comprising a recombinant vector which comprises a nucleotide sequence encoding for expression a mammaglobin B cell antigen and/or a mammaglobin T_c cell antigen.
6. A method for treating a patient with a mammaglobin-expressing tumor which comprises administering to the patient a mammaglobin-specific vaccine comprising an immunogenically-effective amount of a mammaglobin antigen.
7. The method of Claim 6, wherein said antigen is loaded onto a carrier cell.
8. The method of Claim 5, wherein said vaccine also comprises a pharmaceutically acceptable adjuvant.
9. The method of Claim 5, wherein said mammaglobin antigen comprises an amino acid sequence which is encoded for expression by a nucleotide sequence in a recombinant vector.
10. A method for treating a patient with a mammaglobin-expressing breast tumor which comprises isolating mammaglobin-specific lymphocytes from the tumor, activating the lymphocytes with at least one mammaglobin antigen, and administering the activated lymphocytes to the patient.
11. An isolated and purified polypeptide comprising at least one mammaglobin antigen which comprises at least six contiguous amino acids of SEQ ID NO:2, wherein said mammaglobin antigen is recognized by B cells and/or T_c cells specific for a naturally occurring, secreted mammaglobin polypeptide which is glycosylated and consists of amino acids 20-93 of SEQ ID NO:2.
12. The isolated and purified polypeptide of Claim 11, wherein the mammaglobin antigen comprises at least 12 contiguous amino acids of SEQ ID NO:2.

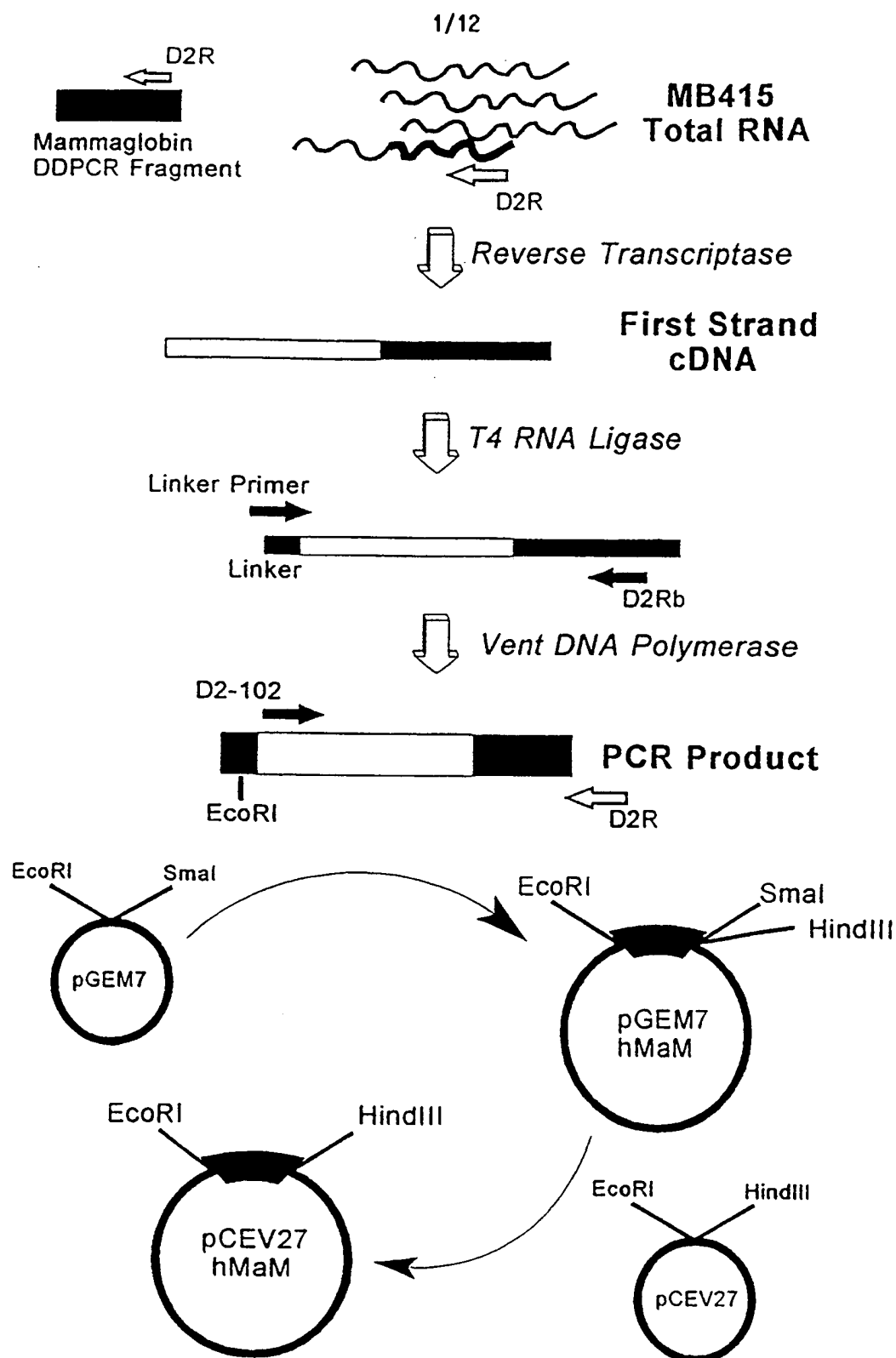


Figure 1

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2/12

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	63	72	81	90	99	108												
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	Met	Lys	Leu	Leu	Met	Val	Leu	Met	Leu	Ala	Ala	Leu	Ser	Gln	His	Cys		
	1																	
	117	126	135	144	153	162												
	TAC	GCA	GGC	TCT	GGC	TGC	CCC	TTA	TTG	GAG	AAT	GTG	ATT	TCC	AAG	ACA	ATC	AAT
	Tyr	Ala	Gly	Ser	Gly	Cys	Pro	Leu	Leu	Glu	Asn	Val	Ile	Ser	Lys	Thr	Ile	Asn
	20																	
	171	180	189	198	207	216												
	CCA	CAA	GTG	TCT	AAG	ACT	GAA	TAC	AAA	GAA	CTT	CTT	CAA	GAG	TTC	ATA	GAC	GAC
	Pro	Gln	Val	Ser	Lys	Thr	Glu	Tyr	Lys	Glu	Leu	Leu	Gln	Glu	Phe	Ile	Asp	Asp
	40																	
	225	234	243	252	261	270												
	AAT	GCC	ACT	ACA	AAT	GCC	ATA	GAT	GAA	TTG	AAG	GAA	TGT	TTT	CTT	AAC	CAA	ACG
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	60																	
	279	288	297	306	315	324												
	GAT	GAA	ACT	CTG	AGC	AAT	GTT	GAG	GTG	TTT	ATG	CAA	TTA	ATA	TAT	GAC	AGC	AGT
	Asp	Glu	Thr	Leu	Ser	Asn	Val	Glu	Val	Phe	Met	Gln	Leu	Ile	Tyr	Asp	Ser	Ser
	80																	
	333	342	351	360	369	378												
	CTT	TGT	GAT	TTA	TTT	TAA	CTT	TCT	GCA	AGA	CCT	TTG	GCT	CAC	AGA	ACT	GCA	GGG
	Leu	Cys	Asp	Leu	Phe	***												
	90																	
	387	396	405	414	423	432												
	TAT	GGT	GAG	AAA	CCA	ACT	ACG	GAT	TGC	TGC	AAA	CCA	CAC	CTT	CTC	TTT	CTT	ATG
	441	450	459	468	477	486												
	TCT	TTT	TAC	TAC	AAA	CTA	CAA	GAC	AAT	TGT	TGA	AAC	CTG	CTA	TAC	ATG	TTT	ATT
	495																	
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Figure 2

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3/12

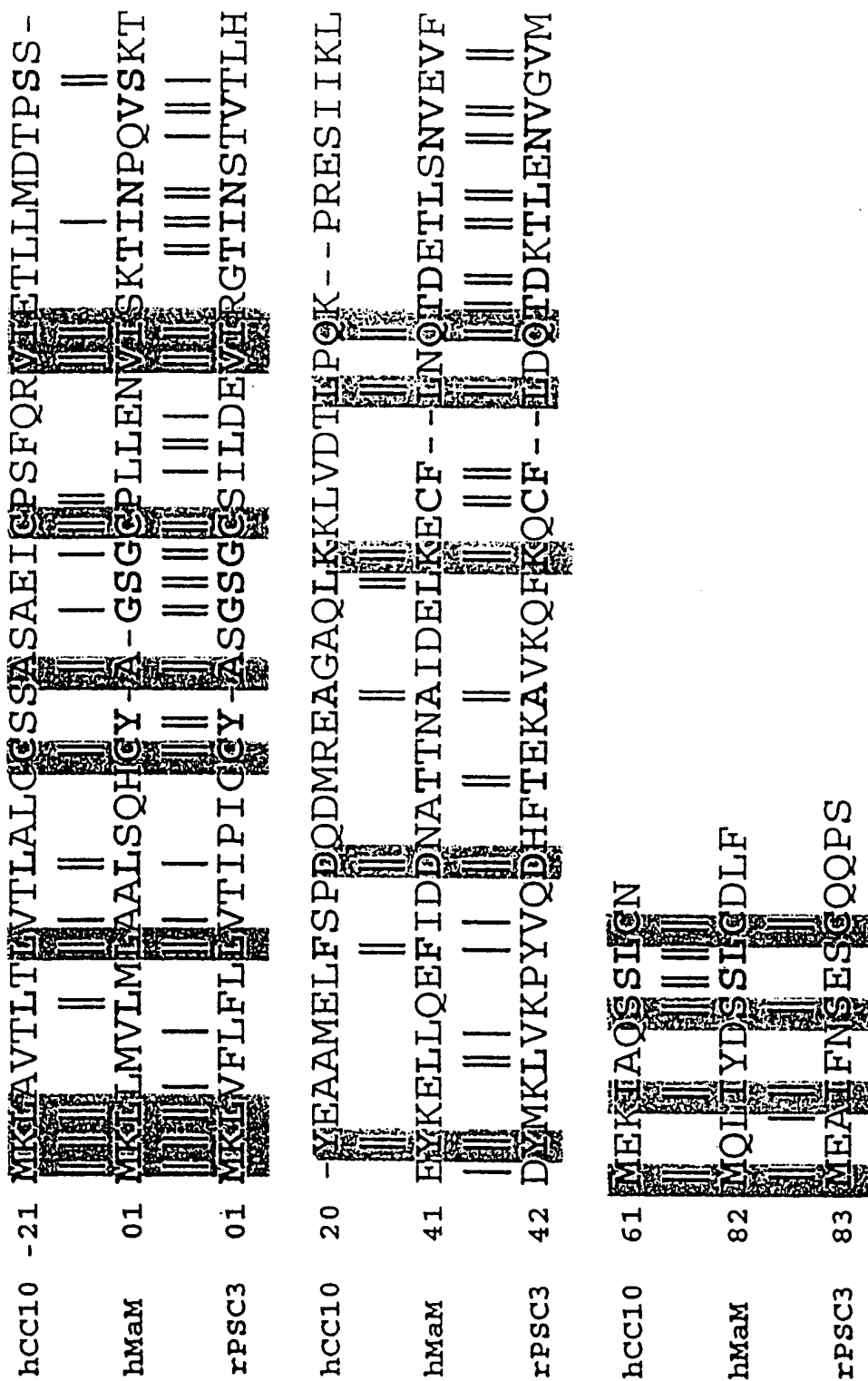


Figure 3

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4/12

Figure 4A

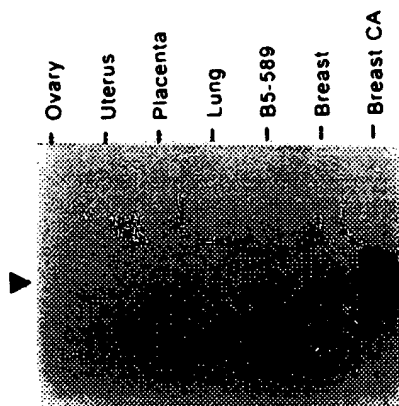
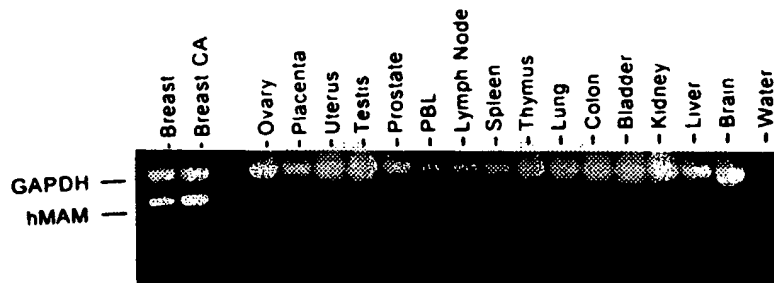
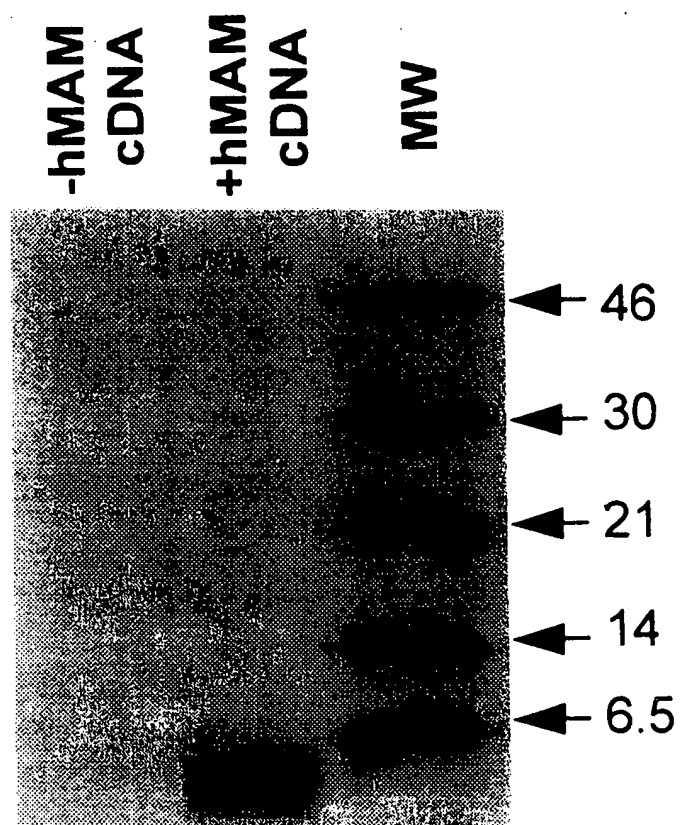


Figure 4B



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5/12

**Figure 5**

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6/12

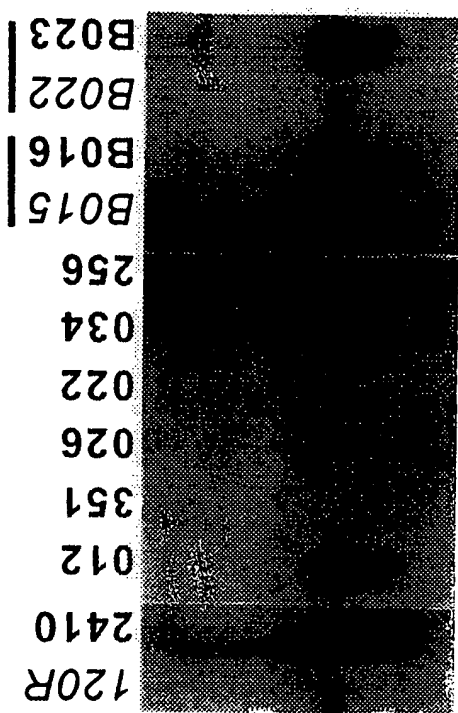


Figure 6

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7/12

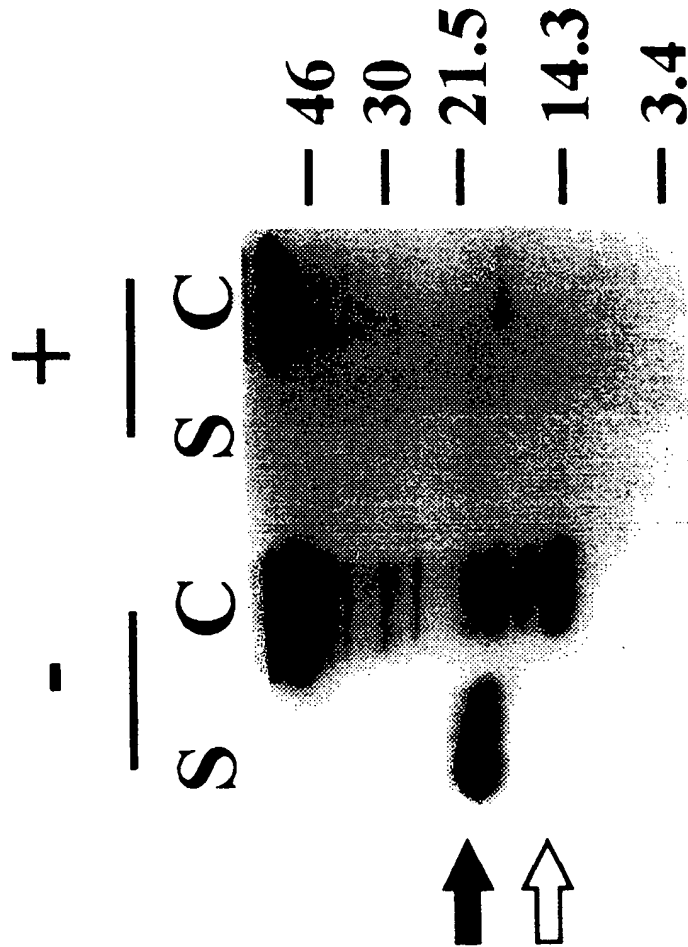


Figure 7

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8/12

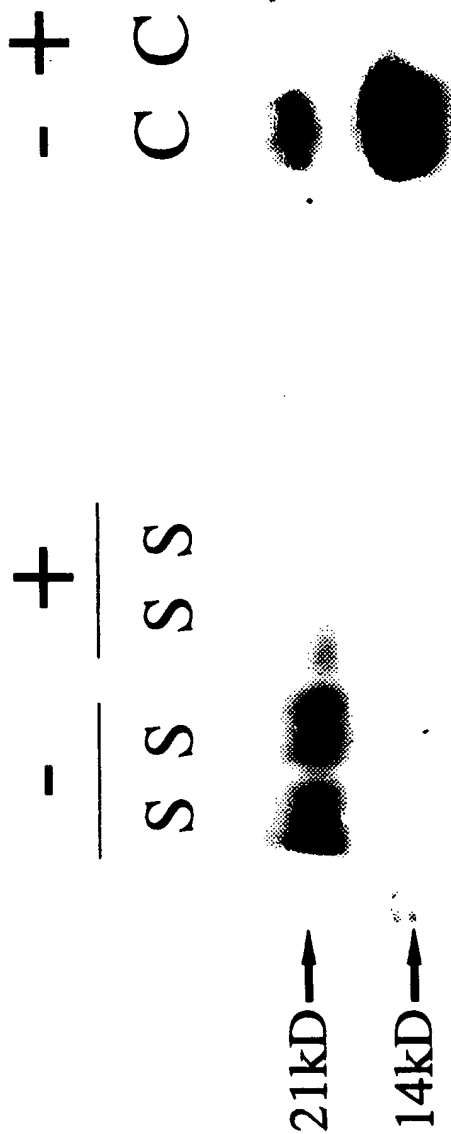


Figure 8

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9/12

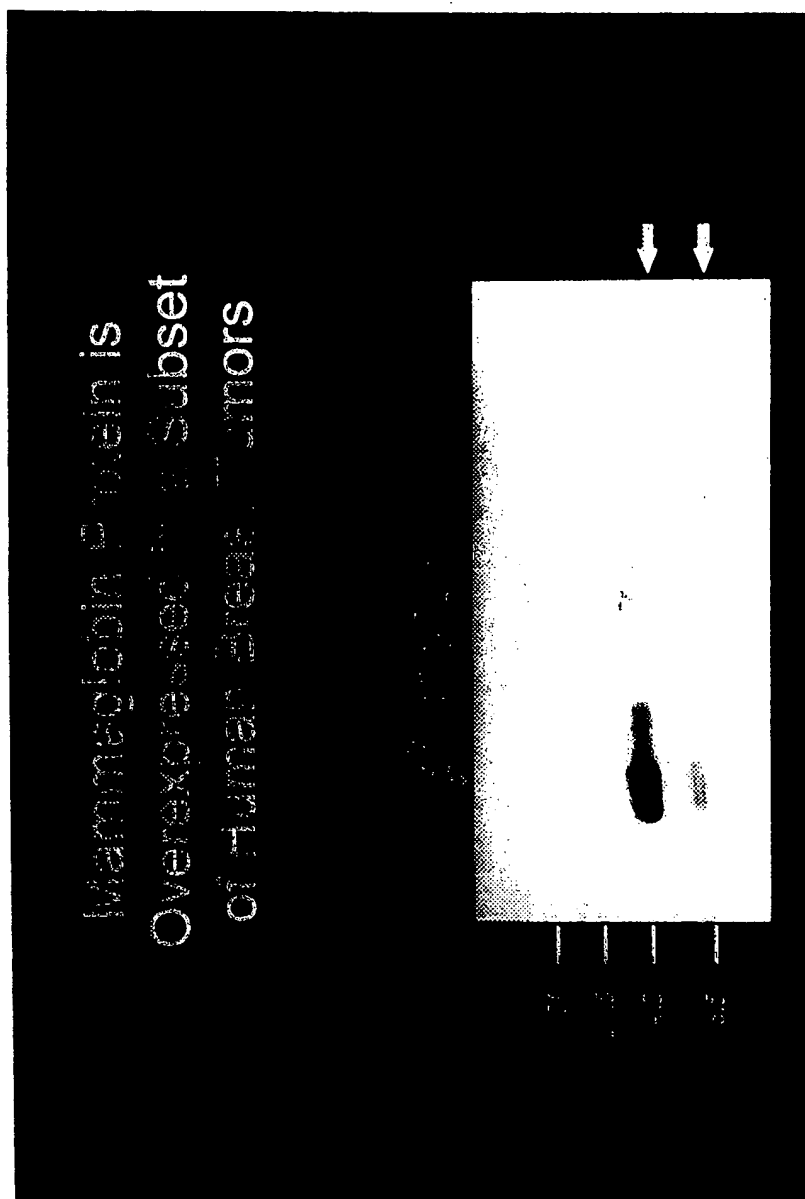
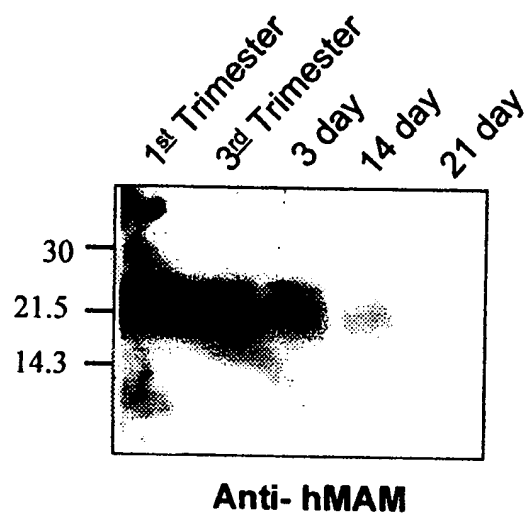


Figure 9

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10/12

Mammaglobin Protein is Secreted from Proliferating Mammary Gland

**Figure 10**

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11/12

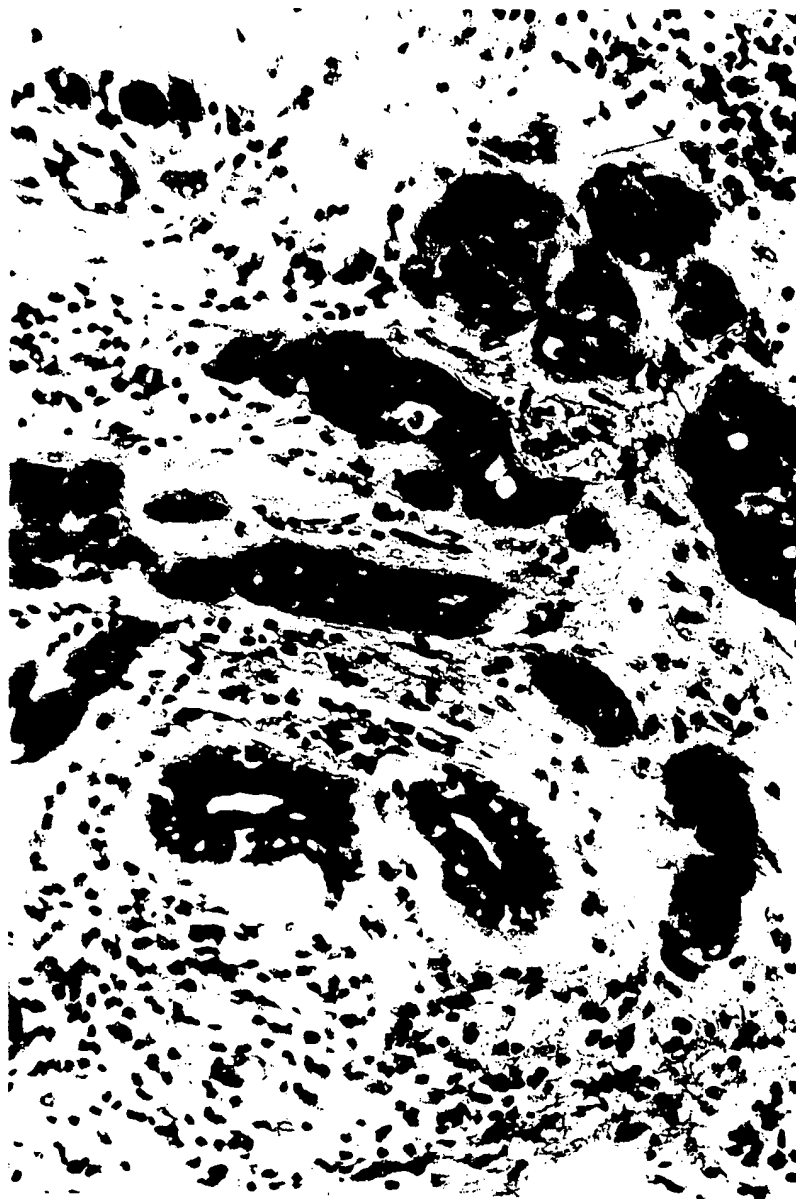


Figure 11A

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12/12

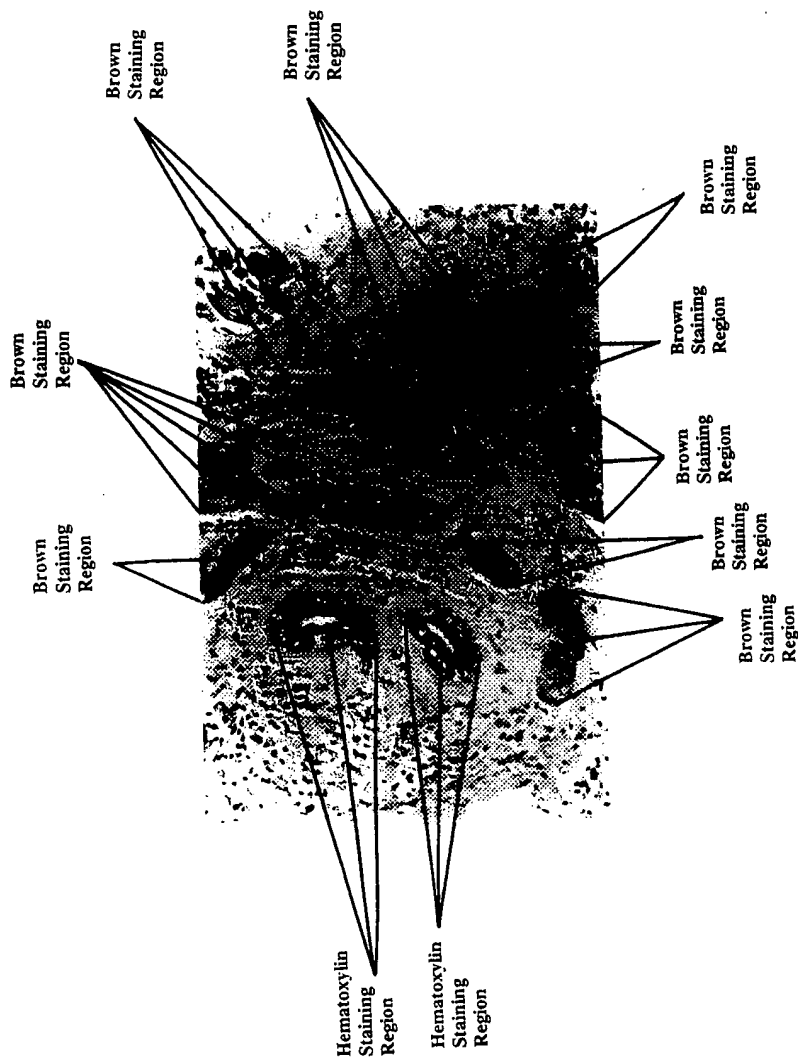


Figure 11B

SUBSTITUTE SHEET (RULE 26)

1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: WASHINGTON UNIVERSITY
- (ii) TITLE OF INVENTION: MAMMAGLOBIN, A SECRETED
MAMMARY SPECIFIC BREAST CANCER PROTEIN
- (iii) NUMBER OF SEQUENCES: 14
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: HOWELL & HAFERKAMP, L.C.
 - (B) STREET: 7733 FORSYTH BOULEVARD, SUITE 1400
 - (C) CITY: ST. LOUIS
 - (D) STATE: MISSOURI
 - (E) COUNTRY: USA
 - (F) ZIP: 63105-1817
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: HENDERSON, MELODIE W.
 - (B) REGISTRATION NUMBER: 37,848
 - (C) REFERENCE/DOCKET NUMBER: 6029-6443
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (314) 727-5188
 - (B) TELEFAX: (314) 727-6092

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 503 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

2

GACAGCGGCT TCCTTGATCC TTGCCACCCG CGACTGAACA CCGACAGCAG CAGCCTCACC
60

ATGAAGTTGC TGATGGTCCT CATGCTGGCG GCCCTCTCCC AGCACTGCTA CGCAGGCTCT
120

GGCTGCCCCCT TATTGGAGAA TGTGATTTCC AAGACAATCA ATCCACAAGT GTCTAAGACT
180

GAATACAAAG AACTTCTTCA AGAGTTCATA GACGACAATG CCACTACAAA TGCCATAGAT
240

GAATTGAAGG AATGTTTTCT TAACCAAACG GATGAAACTC TGAGCAATGT TGAGGTGTTT
300

ATGCAATTAA TATATGACAG CAGTCTTTGT GATTTATTTT AACTTTCTGC AAGACCTTTG
360

GCTCACAGAA CTGCAGGGTA TGGTGAGAAA CCAACTACGG ATTGCTGCAA ACCACACCTT
420

CTCTTTCTTA TGTCTTTTTA CTACAAACTA CAAGACAATT GTTGAAACCT GCTATACATG
480

TTTATTTTAA TAAATTGATG GCA
503

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Leu Leu Met Val Leu Met Leu Ala Ala Leu Ser Gln His Cys
1 5 10 15

Tyr Ala Gly Ser Gly Cys Pro Leu Leu Glu Asn Val Ile Ser Lys Thr
20 25 30

Ile Asn Pro Gln Val Ser Lys Thr Glu Tyr Lys Glu Leu Leu Gln Glu
35 40 45

Phe Ile Asp Asp Asn Ala Thr Thr Asn Ala Ile Asp Glu Leu Lys Glu
50 55 60

Cys Phe Leu Asn Gln Thr Asp Glu Thr Leu Ser Asn Val Glu Val Phe
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Met Gln Leu Ile Tyr Asp Ser Ser Leu Cys Asp Leu Phe
85 90

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGCGGCTTC CTTGATCCTT G
21

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 403 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA to mRNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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120

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(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 206 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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120

CTTCTCTTTC TTATGTCTTT TTA CTACAAA CTACAAGACA ATTGTTGAAA CCTGCTATAC
180

ATGTTTATTT TAATAAATTG ATGGCA
206

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 95 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Tyr Ala Ser Gly Ser Gly Cys Ser Ile Leu Asp Glu Val Ile Arg Gly
      20              25              30
Thr Ile Asn Ser Thr Val Thr Leu His Asp Tyr Met Lys Leu Val Lys
      35              40              45
Pro Tyr Val Gln Asp His Phe Thr Glu Lys Ala Val Lys Gln Phe Lys
      50              55              60
Gln Cys Phe Leu Asp Gln Thr Asp Lys Thr Leu Glu Asn Val Gly Val
 65              70              75              80
Met Met Glu Ala Ile Phe Asn Ser Glu Ser Cys Gln Gln Pro Ser
      85              90              95

```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 91 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met Lys Leu Ala Val Thr Leu Thr Leu Val Thr Leu Ala Leu Cys Cys
 1              5              10              15
Ser Ser Ala Ser Ala Glu Ile Cys Pro Ser Phe Gln Arg Val Ile Glu
      20              25              30
Thr Leu Leu Met Asp Thr Pro Ser Ser Tyr Glu Ala Ala Met Glu Leu
      35              40              45
Phe Ser Pro Asp Gln Asp Met Arg Glu Ala Gly Ala Gln Leu Lys Lys
      50              55              60
Leu Val Asp Thr Leu Pro Gln Lys Pro Arg Glu Ser Ile Ile Lys Leu
 65              70              75              80
Met Glu Lys Ile Ala Gln Ser Ser Leu Cys Asn
      85              90

```

6

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CACGAATTCA CTATCGATTC TGGAACCTTC AGAGG
35

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGGTTCGGC CCACCTCTGA AGGTTCCAGA ATCGATAG
38

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

7

AATCCGTAGT TGGTTTCTCA CC
22

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTTTCTGCAA GACCTTTGGC
20

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTTTTTTT TTTTTTTTTT T
21

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Glu	Val	Phe	Met	Gln	Leu	Ile	Tyr	Asp	Ser	Ser	Leu	Cys	Asp	Leu	Phe
1				5					10					15	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/17991

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 1/00; C07H 21/00; A01N 61/00, 63/00

US CL : 530/350; 536/23/1; 514/1, 2; 424/93/1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23/1; 514/1, 2; 424/93/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PEETERS et al. Structural Studies on Rat Prostatic Binding Protein. European Journal of Biochemistry. 1981, Vol. 115, pages 115-121, see entire document especially Figure 1, page 118.	1-4 and 11-20
Y	US 5,610,031 A (BURGESON ET AL.) 11 March 1997, see col 13, line 65 through col 14 line 25.	1-4 and 11-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 NOVEMBER 1998

Date of mailing of the international search report

02 DEC 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17991

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4 and 11-20

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/17991

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, CABA, CAPLUS, EMBASE, GENBANK, LIFESCI, MEDLINE, SCISEARCH

search terms: uteroglobin, glycosylate, bradykinin, precursor, mammaglobin, antibody

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4 and 11-20, drawn to a mammaglobin-specific vaccine comprising an immunogenically effective amount of at least one mammaglobin antigen which is an isolated and purified polypeptide comprising at least one mammaglobin antigen which comprises at least six contiguous amino acids of SEQ ID NO:2, wherein said mammaglobin antigen is recognized by B cells and/or T_H cells specifically for a naturally occurring, secreted mammaglobin polypeptide which is glycosylated and consists of amino acids 20-93 of SEQ ID NO. 2.

Group II, claim(s) 5 and 9, drawn to a mammaglobin-specific vaccine comprising a recombinant vector which comprises a nucleotide sequence encoding for expression of a mammaglobin B cell antigen and/or a mammaglobin T_H cell antigen.

Group III, claim(s) 6-8, drawn to a method of treating a patient with a mammaglobin expressing tumor comprising administering to the patient a mammaglobin-specific vaccine comprising an immunogenically-effective amount of a mammaglobin antigen.

Group IV, claim 10, drawn to a method for treating a patient with a mammaglobin-expressing breast tumor which comprises isolating mammaglobin-specific lymphocytes from the tumor, activating the lymphocytes with at least one mammaglobin antigen, and administering the activated lymphocytes to the patient.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-IV appears to be that they all relate to a mammaglobin-specific vaccine comprising an immunogenically effective amount of at least one mammaglobin antigen.

However, the vaccine claims are obvious over Peeters et al in view of US Patent No. 5,610,031 since Peeters et al teach a purified and isolated polypeptide comprising two sets of 11 contiguous amino acid residues that are homologous to SEQ ID NO:2 in that they are conservative substitutions of the amino acid sequences recited in SEQ ID NO:2, that is S is substituted for P, I is substituted for L, D is substituted for E, E is substituted for N, Q is substituted for R, R is substituted for L and D is substituted for N and US Patent No. 5,610,031 teaches that it is conventional to prepare analogs of peptides and that preferred analogs include those whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions.

Therefore, the technical feature linking the inventions of groups I-IV does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a vaccine comprising at least one mammaglobin antigen.

The special technical feature of Group II is a mammaglobin-specific vaccine comprising a nucleotide sequence encoding a mammaglobin B cell antigen and/or a mammaglobin T_H cell antigen.

The special technical feature of Group III is a method of treating a patient with a vaccine containing a mammaglobin antigen.

The special technical feature of Group IV is a method for treating a patient comprising isolating mammaglobin-specific lymphocytes from the tumor.

Accordingly, Groups I-IV are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.